Influences of nutrient limitation on Bacillus species

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Influences of Nutrient Limitation on Bacillus Species

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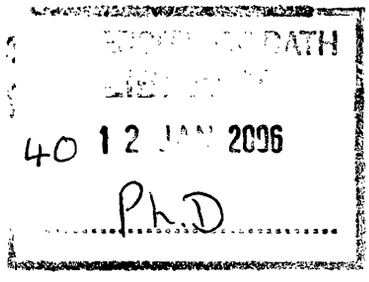
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Abstract

Due to the recent bioterrorism attacks, there has been an increased interest in the study of anthrax. These studies have focused on *Bacillus anthracis* physiology and the development of improved anthrax vaccines. The current UK anthrax vaccine is prepared from cell free supernatants of *B. anthracis* cultures that contains the anthrax toxin components; protective antigen, oedema factor and lethal factor. Investigations into the anthrax vaccine growth medium have revealed a requirement for carbon starvation, pellicle formation and the addition of charcoal to the medium in order to produce maximal vaccine yield. In addition, it has also been proposed that the vaccine medium leads to phosphate limited growth of *B. anthracis*. Since previous studies have also shown that nutrient limitations can affect toxin production and sporulation of Bacilli, the environmental conditions arising in the vaccine medium are also likely to influence cell physiology, toxin and protease production, explaining variations in yield arising in the final vaccine.

This project has therefore developed a medium for studies into the effects of nutrient limitation on cell physiology of the related bacterium, *B. cereus*. This derived medium enabled growth limitation of defined nutrients allowing the effects of specific nutrient limitation on planktonic growth to be studied. These studies revealed that nutrient limitation influences heat resistance, sporulation, toxin and protease production of *B. cereus*. In addition the influence of biofilm formation on toxin production was also investigated and studies have shown that toxin production also occurs in *B. cereus* biofilms. Furthermore, the chemically defined medium derived for *B. cereus* has also been used to culture *B. anthracis* under varying nutrient limitations and toxin assays have been completed on the culture supernatants. These data show that *B. anthracis* and *B. cereus* have varying nutrient requirements, demonstrating that *B. cereus* cannot be used as an accurate surrogate for *B. anthracis* in all situations.

Key Words; *B. cereus*, *B. anthracis*, nutrient limitation, toxin production, proteases and biofilms.

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Abbreviations

σ^B	alternative sigma factor B
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pairs
°C	degrees centigrade
CDM	chemically defined medium
cfu	colony forming units
CO_2	carbon dioxide
CT	crossing threshold
DTPA	diethylenetriaminepenta acetic acid
EDTA	ethylenediamine tetra-acetic acid
EF	oedema factor
ELISA	enzyme linked immunosorbent assay
EPS	exopolysaccharide
ET	oedema toxin
DNA	deoxyribonucleic acid
GSP	general stress proteins
GSR	general stress response
HBL	haemolysin BL
HPA	Health Protection Agency
HRP	Horseradish peroxidase
Kb	kilo base
kDa	kilo Daltons
LB	Luria-Bertani medium
LF	lethal factor
LT	lethal toxin
µl, ml, l	microlitre, millilitre, litre
µM, mM, M	micromolar, millimolar, molar
mRNA	messenger RNA

NCBI	National Centre for Biotechnology Information
Nhe	non-haemolytic enterotoxin
nm, µm, mm, cm, m	nanometre, micrometre, millimetre, centimetre, metre
OD	optical density
PA	protective antigen
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg / ng / µg / mg / g	picogram, nanogram, microgram, milligram, gram
PIC	protease inhibitor cocktail
PMSF	phenyl methyl sulfonyl fluoride
ppm	parts per million
PVC	polyvinylchloride
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
SD	standard deviation
SDM	semi defined medium
sec, min, h, d	seconds, minutes, hours, days
SEM	standard error of the mean
UV	ultra violet
w/v	weight / volume

Chapter 1 - Introduction

1.1 Overview

In 2001, *Bacillus anthracis*, the causative agent of anthrax, was used for the first time in an act of bioterrorism (Mourez *et al.*, 2002). This act has led to a renewed interested in the bacteria and its properties. Anthrax has, however, been previously studied for many years and has a reasonable degree of historical significance. Natural outbreaks of the disease in domestic animals in the late 1800's instigated the initial studies into this bacterium and research was renewed during World War II when *B. anthracis* was studied as an effective biological warfare weapon (Spencer, 2003; Turnbull, 2002). It was also highlighted in 1979 when *B. anthracis* spores were accidentally released from a Soviet military research facility (Meselson *et al.*, 1994).

In addition to these outbreaks, anthrax has been recognised for centuries as a serious disease in both animals and humans. It has inflicted numerous agricultural losses and it used to be a common infection in mill workers who handled the hides of infected animals (Baillie, 2001; Spencer, 2003; Swartz, 2001; Turnbull, 2002). For this reason, vaccines against anthrax were introduced for both cattle and for humans that were considered to be at risk (Friedlander *et al.*, 2002; Shlyakhov and Rubinstein, 1994; Turnbull, 1991). These vaccines are still in production and with the renewed threat of *B. anthracis* being used as a biological weapon, are still clinically important.

The current human vaccine has been in production in the United Kingdom for over 40 years. It is produced by the Health Protection Agency at Porton Down and consists of alum precipitated anthrax toxins produced from an attenuated strain of *B. anthracis* (Baillie, 2001; Friedlander *et al.*, 2002; Turnbull, 1991). Whilst this vaccine has been in production for many years and is believed to be effective (Demicheli *et al.*, 1998), little research has been completed to understand fully the kinetics of toxin production during the process and the gross physiology of the *B. anthracis* cells used.

It is known that during growth of *B. anthracis* for vaccine production, cells encounter stress conditions that are likely to influence final toxin production. These stresses include a potential phosphate starvation leading to low levels of growth and an imposed oxygen limitation due to the cultures being statically incubated. The static conditions also allow the cultures to form a pellicle on the surface of the flasks and in addition, charcoal is added to the media allowing the possibility of biofilm growth on the charcoal granules. It is unknown if these features have an effect on the overall vaccine yield. In addition the toxins are harvested when there is an observed drop in culture pH, this is believed to co-incide with maximal toxin production, but the full kinetics of this process are still unclear (Personal communication – Richard Sharp, HPA).

Overall, it is clear that there are a number of factors that may influence toxin production in the vaccine procedure, leading to inconsistencies in the final vaccine in terms of toxin yields and ratios. For this reason further research is required to optimise vaccine production in order to enhance reproducibility and increase yield. In order for this to be completed a greater understanding of *Bacillus anthracis* physiology under stress conditions, including nutrient and oxygen limitation, is required.

Unfortunately, there are a number of constraints for working with *B. anthracis* (due to requirement of appropriate facilities and licensing), therefore *B. cereus* has been used as a surrogate for *B. anthracis*. This bacterium is closely related to *B. anthracis* (see section 1.2.1) and therefore it is likely that the effects of nutrient and oxygen limitation on *B. cereus* are similar to the effects on *B. anthracis*. Hence the study of *B. cereus* physiology under stress conditions should also lead to a greater understanding of *B. anthracis* physiology. Furthermore, *B. cereus* has also been described as a human food pathogen (see section 1.5) and therefore studies into the effect of environmental stresses on *B. cereus* may also lead to a greater understanding of food-borne infections.

1.2 *Bacillus*

The *Bacillus* species are found ubiquitously in the environment and are Gram-positive or occasionally Gram-variable bacteria. They are either aerobic or facultatively anaerobic and have the ability to form endospores. The vegetative cells are either square, round or straight-ended rods and can occur both as single cells and as long chains. Depending on the strain, the cells can range in size between 0.5 by 1.2 μm to 2.5 by 10 μm in diameter. Bacilli grow under optimal temperatures ranging between 25 to 37°C, however thermophilic and psychrophilic members of the group can grow at extreme temperatures as low as 3°C and up to 75°C. In addition some members of the genus are capable of growing under extreme acidity or alkaline conditions, between pH 2-10. With the exception of *B. anthracis* and *B. myocides*, all bacilli are motile and the majority of the *Bacillus* species are catalase positive. Some members of the *Bacillus* genus including *B. anthracis, B. subtilis* and *B. megaterium* are also capable of forming a polypeptide capsule under appropriate conditions. The G+C content of the DNA varies in the genus between 32-69% (Drobniewski, 1993; Turnbull and Kramer, 2001).

When *Bacillus* cells are deprived of essential nutrients, in the presence of oxygen, they can undergo sporulation. The resulting spores can then persist in a dormant state in the soil for many years resisting extreme stress conditions (Henriques and Moran, 2000). Depending on the strain, the final endospores can be cylindrical, oval, round or kidney-shaped with terminal, sub terminal or central swelling of the sporangium (Turnbull and Kramer, 2001). When these spores encounter certain environmental signals, they can undergo germination. This process is regulated by a number of germination operons in the cell and once completed, the resulting vegetative cell has a restored active metabolism and is capable once again of replication and the production of a range of virulence factors (Barlass *et al.*, 2002; Weiner and Hanna, 2003).

Research into the spores of *B. anthracis* and *B. cereus* has shown them to be enclosed by a prominent loose fitting layer called the exosporium. The full molecular structure

and function of the exosporium is not well characterised, but is believed to act as a primary permeability barrier, preventing exoenzymic attack on the spore coat and cortex layers (Steichen *et al.*, 2003).

Bacilli are mainly saprophytes and are widely distributed. They are commonly found in the soil and are spread in dust, water and via plant and animal materials. The *Bacillus* genus include a number of mesophilic, thermophile, pyschrophile, acidophile and halophile strains, hence, due to this range in physiology and the ability of the family to form spores, members are able to survive and even grow under a variety of extreme conditions (Turnbull and Kramer, 2001).

The majority of the *Bacillus* genus are not clinically significant and have no pathogenic potential, however the notable exceptions to this are *B. anthracis* leading to anthrax infection and *B. cereus*; a common food poisoning agent. In addition *B. thuringiensis* has also been implicated in food poisoning syndromes (Damgaard *et al.*, 1996; Drobniewski, 1993; Little and Ivins, 1999).

Generally the *Bacillus* genus are easy to identify since they are mainly Gram-positive spore formers that are catalase positive. However due to the pathogenic nature of *B. anthracis* and *B. cereus*, further identification procedures have been developed for these bacteria. *B. anthracis* is non-fastidious and grows well on simple media, it forms characteristic colonies that include a medusa head and growth under elevated CO_2 encourages the formation of the capsule and smooth colonies. *B. anthracis* smears can be identified using the McFadyean reaction and it is also non-haemolytic on horse blood agar. Generally, biochemical reactions are of no use in identifying the bacteria. *B. cereus* also has a number of identifiable characteristics; like *B. anthracis* it is non-fastidious, however unlike *B. anthracis,* it is capable of haemolysis on horse and sheep blood agar plates. Other chemical markers are the ability to produce lecithinase and the inability to utilise mannitol (Anderson *et al.* 1998; Drobniewski, 1993; Swartz, 2001; Turnbull and Kramer, 2001). Further comparisons between B. *anthracis*, *B. thuringiensis* and *B. cereus* are listed in Table 1.1.

	B. anthracis	*B. thuringiensis*	*B. cereus*
Phenotype			
Penicillin resistance	11% of strains tested resistant to Penicillin G	Yes	1 % of strains tested showed no resistance to ampicillin
Haemolytic activity (on sheep erythrocytes)	No – however weak haemolysis by some strains has been reported	Yes	Majority of strains
Mucoid colony (capsule synthesis)	Yes	No	No
Crystalline parasporal inclusions	No	Majority of strains	No
Motility	No – however isolated monoflagella isolates have been reported	Majority of strains	Majority of strains
Genotype			
pX01	Yes	Analogous sequence homology reported	Highly homologous sequence reported
pX02	Yes, however plasmid can be easily lost	Analogous plasmid reported	
Phospholipase C	+	+	+
nheA gene	+	+	+
Ecology			
Host range (toxin specific)	Vertebrates	Invertebrates. Specific toxins only active against a limited number of invertebrate hosts	Not Known
Distribution	Worldwide (many areas not yet studied)	Worldwide (not isolated in Antarctica)	Worldwide
Prevalence in hosts	Endemic in Africa and Asia	Generally low levels of infection, occasional epidemics among mosquitoes and insects in stored product environments	Present in invertebrates but not considered a disease of invertebrates

Table 1.1 - phenotypic, genotypic and ecological features of *B. cereus*, *B. thuringiensis* and *B. anthracis* (Adapted from Jensen *et al.*, 2003).

1.2.1 *B. cereus* group

The *B. cereus* group is a homogeneous cluster within the *Bacillus* family; consisting of *B. anthracis, B. cereus, B. myocides, B. pseudomycoides, B. weihenstephanensis* and *B. thuringiensis.* Although they exhibit widely different phenotypes and pathogencity, it has been suggested that a number of the bacteria should be considered members of the same species based on their genomic sequences (Helgason *et al.*, 2000), however other data imply that they are genetically distinct species (Radnedge *et al.*, 2003; Ticknor *et al.*, 2001). These conflicting results are problematic in terms of taxonomy, however, despite the inconsistent results it is clear that the close relationships of the members of the *B. cereus* group are important in terms of virulence factors and gene transfer (Helgason *et al.*, 2000).

Studies into the genetic relationship between *B. anthracis* and *B. cereus* have shown the two bacteria to share high homology. Sequence identities average at 96.5% at the amino acid level, depending on the strain used, with the DNA levels being similar (Helgason *et al.*, 2000). Due to the high level of similarity between chromosomal DNA, it has been claimed that the species are functionally distinguished by the genes carried on the plasmids.

The *B. anthracis* genome has been sequenced and estimated to have a 33% G+C content (Mock and Fouet, 2001; Okinaka *et al.*, 1999; Read *et al.*, 2003). The genome comprises of the chromosome and two plasmids, pX01 and pX02. Several chromosomally encoded proteins are believed to contribute to the pathogencity of the *B. anthracis*, including haemolysins, phospholipases and iron acquisition functions, however, the main *B. anthracis* virulence factors are carried on the plasmids. The presence of both plasmids have been shown to be required for full virulence, although the plasmids alone are not capable of virulence since some *Bacillus* strains that carry both plasmids are avirulent (Keim and Smith, 2002). Curing of the pX01 plasmid is rare, however pX02 is easily and spontaneously lost (Mock and Fouet, 2001). pX01 harbors the toxin genes *pagA*, *cya* and *lef*, that encode protective antigen, lethal factor

and oedema factor respectively. These genes are located on a pathogencity island that is flanked by inverted IS*1627* elements, the presence of which suggests that the pathogencity island was transposed into the pX01 plasmid (Okinaka *et al.*, 1999).

The pX02 carries the three genes essential for the biosynthesis of the poly-γ-D-glutamic acid capsule; *capB*, *capC* and *capA*. These genes have been shown to encode for membrane associated enzymes of molecular weights 44.8, 16.5 and 46.4 kDa, respectively (Makino *et al.*, 1989, Uchida *et al.*, 1985). An additional protein, with a predicted size of 51 kDa, is encoded by the *dep* gene that is located downstream of the cap region. This protein appears to be a depolymerase that catalyses the hydrolysis of poly-γ-D-glutamic acid into lower molecular weight polyglutamates (Ezzell and Welkos, 1999; Mock and Fouet, 2001; Uchida *et al.*, 1993a).

The *B. cereus* strain ATCC 10987 has recently been sequenced and been shown to have greater similarity to *B. anthracis* Ames than to the *B. cereus* type strain ATCC 14579 used in this study. *B. cereus* ATCC 10987 also harbours a single large plasmid (pBc10987), that is similar in gene content and organisation to the *B. anthracis* plasmid pX01; however this plasmid lacks the pathogencity associated island containing the anthrax toxin genes found in pX01. These data further highlight the genetic relationship between the strains and the potential for gene transfer (Rasko *et al.*, 2004). In addition, further studies have also identified a *B. cereus* strain harbouring the plasmid pBCX01 that has a 99.6% similarity to the pX01 plasmid of *B. anthracis*. Although a pX02 plasmid has not been found in *B. cereus*, the plasmid pBC218, has been identified in one strain and has been shown to encode a polysaccharide capsule (Hoffmaster *et al.*, 2004). This strain of *B. cereus* was also associated with an illness resembling inhalational anthrax.

1.3 *B. anthracis*

B. anthracis is the causative agent of anthrax and as such is a category 3 organism (Advisory Committee in Dangerous Pathogens). All mammals are susceptible to *B. anthracis*, however anthrax is primarily a disease of herbivores (Mock and Fouet, 2001).

1.3.1 Disease

The disease is initiated by the entry of spores into the host; this can either be cutaneously through cuts or through an insect bite, or systemically via ingestion or inhalation. Therefore there are three forms of anthrax infection – cutaneous, gastrointestinal and inhalational and all forms can progress to fatal systemic anthrax (Mock and Fouet, 2001).

1.3.1.1 Cutaneous infection

Over ninety-five percent of naturally occurring anthrax cases are cutaneous and infection in humans normally occurs on the arms, face or neck. Infection is usually noticeable between 1-7 days with the appearance of a painless papule that then continues to develop into an ulcer covered by a characteristic black eschar. The lesions are also always accompanied by substantial oedema. The eschar then dries and falls off within two weeks to leave little permanent scarring. Low grade fever and malaise are often associated with infection, however bacteraemia is a rare complication. Without antibiotic treatment, mortality rates are still as high as 20 percent, however use of appropriate antibiotics decreases the mortality rate to less than 1 percent (Spencer, 2003; Swartz 2001).

1.3.1.2 Gastrointestinal anthrax

Gastrointestinal anthrax symptoms appear around 2-5 days after ingestion of *B. anthracis* endospores, usually from contaminated meat. Few cases of this form of anthrax infection have been reported outside Africa and Asia. Ulceration is always seen in gastrointestinal cases and microscopical examination of tissues reveals massive oedema and mucosal neurosis in infected sites. Gastrointestinal anthrax

infection falls into 2 clinical forms; abdominal and oro-oesophageal anthrax. With the abdominal infection associated symptoms include fever, nausea, vomiting and diffuse abdominal pain. There have also been reports of both diarrhoea and constipation. Morbidity is due to blood loss, fluid and electrolyte imbalance leading to subsequent shock. Death then results from intestinal perforation and anthrax toxaemia. In the cases where the patient survives the symptoms tend to subside after 10-14 days. With oro-oesophageal anthrax, symptoms include sore throat, dysphasia, fever and oedema. If an early diagnosis is made for cases of gastrointestinal anthrax infection, then patients can be cured; unfortunately, diagnosis is difficult due to the non-descriptive nature of the symptoms, leading to a high mortality rate (Dixon *et al.*, 1999; Spencer, 2003).

1.3.1.3 Inhalational anthrax

Naturally occurring inhalational anthrax is rare and usually occurs after the inhalation of endospores from contaminated animal hides. In the 1960's mill workers were regularly exposed to high concentrations of spores, but since the introduction of hygienic measures, including vaccination, cases have decreased (Dixon *et al.*, 1999; Spencer, 2003). The largest outbreak of inhalational anthrax in the 20th century was after the accidental release from a military factory in Sverdlovsk (Meselson *et al.*, 1994). The incubation time for inhalational anthrax is believed to be around 10 days, however the onset of symptoms has been reported as late as 6 weeks post exposure. Initial symptoms are fever, non-productive cough, myalgia and malaise, often resembling viral upper respiratory tract infections (Dixon *et al.*, 1999). As with gastrointestinal infections, therapeutic intervention against inhalational anthrax infections must be initiated early as systemic anthrax infections are nearly always fatal (Little and Ivins, 1999).

Although the lungs are the initial point of contact in inhalational anthrax, they rarely show signs of infection. This is because once *B. anthracis* spores have been inhaled they are phagocytosed into macrophages via the recruitment of F-actin. Once inside the macrophages, the *B. anthracis* spores are then transported to the regional lymph

nodes and the systemic system, germinating on route (Guidi-Rontani *et al.*, 1999a; Guidi-Rontani, 2002; Hanna and Ireland, 1999). This germination process is believed to involve the germination operon *gerX*. This has been shown to have a key role in *B. anthracis* germination, but is also believed to encode for a number of other virulence factors that contribute to the overall pathogencity of *B. anthracis* (Guidi-Rontani *et al.*, 1999b).

The germination of the endospores is closely followed by expression of virulence factors, including the toxin genes. Currently, there is conflicting data onto the exact mechanism of multiplication and escape of *B. anthracis* cells from the macrophages. It has been reported that the anthrax toxins exhibit effects on the macrophages before the vegetative cells can escape and replicate (Guidi-Rontani *et al.*, 1999a; Guidi-Rontani *et al.*, 2001); however other reports suggest that it is the presence of other genes and virulence factors and not the anthrax toxins that are required for increased macrophage permeability and the escape of *B. anthracis* cells (Dixon *et al.*, 2000).

When the mature vegetative cells escape from the alveolar macrophages, they multiply in the bloodstream at an increased rate. The bacteria are capable of reaching concentrations of approximately 10^8 organisms / ml, and eventually lead to bacteraemia (Hanna and Ireland, 1999). The released toxins can then bind to other host cells leading to toxaemia (Dixon *et al.*, 2000).

1.3.1.4 Anthrax meningitis

Anthrax meningitis is a rare complication when there is involvement of the meninges. Anthrax meningitis occurs in gastrointestinal and inhalational cases of anthrax infection and is nearly always fatal, with death occurring within 1-6 days of infection, despite treatment with antibiotics. In the rare cases where patients have survived, the antibiotic therapy has been combined with antitoxin, prednisone, or both. Symptoms are similar to common meningitis with fever, fatigue, myalgia, headache, nausea, vomiting and occasionally seizures and delirium. Initial signs are followed by rapid

neurological degeneration and death. The cerebrospinal fluid is often bloody and contains numerous bacilli (Dixon *et al.*, 1999).

1.3.2 Capsule

B. anthracis is capable of producing a number of virulence factors and a major one is the production of a poly-γ-D-glutamic acid capsule. The capsule enhances virulence by inhibiting phagocytosis of the organism when it is in its vegetative state (Ezzell and Welkos, 1999; Makino *et al.*, 1989; Zwartouw and Smith, 1955). The capsule is also only weakly immunogenic causing a reduced host cell immune response (Chabot *et al.*, 2004). The Dep protein, responsible for the depolymerisation of the capsule, may also add to the virulence of the organism through the production of low molecular weight polyglutamates (Ezzell and Welkos, 1999; Makino *et al.*, 2002). The capsule production is enhanced in the presence of CO_2 / bicarbonate or serum (Meynell and Meynall, 1964). The molecular weights of the polyglutamic chains are between 20-55 kDa in vitro and are estimated to be 215 kDa in vivo (Mock and Fouet, 2001). Isolates that lack capsule are generally reduced in virulence and have therefore been used as vaccine strains (Friedlander *et al.*, 2002). In addition, factors that influence capsule formation appear to be important in determining the final outcome of infection. In susceptible animals, the bacilli remain encapsulated where as in resistant animals the capsule disappears before the bacilli degenerate (Little and Ivins, 1999).

1.3.3 Anthrax toxins

The other major virulence factors produced by *B. anthracis* are the two AB-type toxins, known as lethal toxin (LT) and oedema toxin (ET). These toxins are made of an A-subunit called protective antigen (PA), the cell-binding domain, and an enzymatic B-subunit of either lethal factor (LF) or oedema factor (EF).

1.3.3.1 Protective Antigen

PA was termed due to its ability to elicit a protective immune response against infection and has been shown to have no toxin activity alone without the presence of

either LF or EF (Lacy and Collier, 2002). PA is the most characterised component of the anthrax toxins and was the first to be identified, cloned and sequenced. It is also the easiest component to produce in large amounts (Bhatnagar and Batra, 2001). The mature PA protein (PA83) is 735 amino acids (83 kDa) and shares sequence homology to a family of binary ADP-ribosyltransferase toxins (Lacy and Collier, 2002). The crystal structure for monomeric PA83 has been determined at 2.1Å and shows that it is composed of mainly β-sheets and folds into four functional domains. Each domain is required for the various steps of the intoxication process (Petosa *et al.*, 1997).

1.3.3.1.1 Receptor binding

In order for the anthrax toxins to exert their functional effects, they must be capable of entry into the host cells. This internalisation is a multi-step process that is initiated by the binding of PA to the cell receptors (as shown in figure 1.1). Research has shown that PA binds to the receptors via domain IV, the carboxyl terminus (Brossier *et al.*, 1999; Rosovitz *et al.*, 2003; Singh *et al.*, 1991). Currently two anthrax toxin receptors have been identified, these are Capillary Morphogenesis Protein 2 (CMG2) and Anthrax Toxin Receptor (ATR), also known as TEM8 (Tumour Endothelial Marker 8) (Bradley *et al.*, 2001; Scobie *et al.*, 2003).

ATR is a 368 amino acid, type I protein, and has been shown to be expressed in a wide variety of tissues, but at increased levels in colon tumor vasculature. Original analysis of CMG2 data indicated that it was expressed only in human placenta tissue, however data from the NCBI database suggest that it is also expressed in a wide variety of tissues (Bradley *et al.*, 2001; Scobie *et al.*, 2003). Anthrax toxin receptors have been seen to be widespread, based on the ability of most cell lines tested to translocate EF and LF proteins in a PA dependant manner (Bradley and Young, 2003).

Both of these receptors consist of a single polypeptide chain that includes an extracellular domain, a membrane spanning region and a cytoplasmic tail. In the

extracellular domain of both proteins there is a 200 amino acid sequence comprising an von Willebrand type A (VWA) domain – the amino acid homology of this domain between the two proteins is 60%, and the homology between the whole proteins is 40%. Many VWA domains contain a highly conserved Metal Ion-Dependant Adhesion Site, that is often involved in ligand interactions (Scobie *et al.*, 2003). Consistent with this, both TEM8 and CMG2 have been found to bind PA with a stronger affinity in the presence of divalent cations, and EDTA has been shown to disrupt the interaction (Bradley *et al.*, 2001). The crystal structure of CMG2-PA has recently been resolved (Santelli *et al.*, 2004), as has the VWA domain of CMG2 (Lacy *et al.*, 2004).

1.3.3.1.2 Cleavage, pore formation and translocation

Domain I of PA is comprised of residues 1-258 (Bhatnagar and Batra, 2001). Studies have shown upon the binding of PA to the cell receptor, or prior to receptor binding, domain I can be cleaved by furin-like proteases to yield two subunits; PA63 and PA20 (Brossier *et al.*, 2000; Ezzell and Abshire, 1992, Gordon *et al.*, 1995; Klimpel *et al.*, 1992). This enzymatic cleavage allows the release of the PA20 subunit, relieving the steric hindrances that block EF and LF from binding. Hence cleavage of PA allows EF and LF binding, with the putative binding site being reported as residues 168-258 in domain I of PA (Petosa *et al*, 1997).

Other studies have shown that after receptor binding and cleavage, oligomerisation of PA63 occurs to form ring-shaped heptamers (Milne *et al.*, 1994). Crystal structures of these heptameric prepores have indicated that monomer-monomer interactions within the heptamers are due to residues in domains II and III of PA (Mogridge *et al.*, 2001; Petosa *et al.*, 1997). This oligomerisation of PA was originally reported to allow the binding of 7 LF molecules (Singh *et al.*, 1999), however more recent reports have shown that steric hindrance only allows the binding of up to 3 molecules of LF and / or EF per heptamer (Cunningham *et al.*, 2002). In addition, other studies have shown that EF and LF only bind stably to PA63 dimers or higher order oligomers and not monomeric PA63 (Mogridge *et al.*, 2002). Studies have also shown that cleavage and

oligomerisation of PA triggers endocytosis, since a furin resistant mutant of PA remains at the cell surface for a prolonged period and conditions that inhibit endocytosis also block toxin activity (Beauregard *et al.*, 2000; Gordon *et al.*, 1988).

After endocytosis, the toxins are believed to traffic to the endosome where cation-selective pore formation occurs (Friedlander, 1986). Studies into anthrax toxin pore formation have shown that these pores can form in planar phospholipid bilayers (Blaustein *et al.*, 1989), mammalian cells and liposomes (Koehler and Collier, 1991; Milne and Collier, 1993). Other studies have also shown that the conversion from the pre-pore to the active pore is also via an acid dependant step since studies that changed the trans-membrane gradient of H^+ ions or raised the pH of endocytotic vesicles protects the cells from anthrax toxin (Friedlander, 1986; Gordon *et al.*, 1988). In addition further studies have proposed that this conversion from pre-pore to pore involves domain II of the PA subunits by the insertion of a chymotrypsin sensitive loop into the membrane (Petosa *et al.*, 1997).

It is believed that pore formation allows the translocation of EF and LF to the cytosol, however, in reality it is not clear if pore formation is a prerequisite or a consequence of translocation, since mutants deficient in translocation are also unable to form pores (Sellman *et al.*, 2001a). The exact mechanisms of translocation are also not currently known, the enzymatic LF and EF units may have an active role in their translocation, but cannot translocate without PA63 (Kochi *et al.*, 1994). It is also unclear if translocation occurs through the pore lumen or at the protein lipid interface (Lacy and Collier, 2002). It is likely that EF and LF have to at least partially unfold for their translocation since the diameter of the pore lumen is too narrow for the folded proteins (Klingenberg and Olsnes, 1996; Wesche *et al.*, 1998). It has also been stated that whilst LF is fully translocated into the cytoplasm, EF remains membrane bound, exposing its catalytic domains to the cytosolic compartment. This therefore suggests that EF and LF have differing mechanisms of translocation (Guidi-Rontani *et al.*, 2000). Once translocation has been completed for either LF or EF, the enzymatic units are then able to exert their functions on the cell within the cytosol.

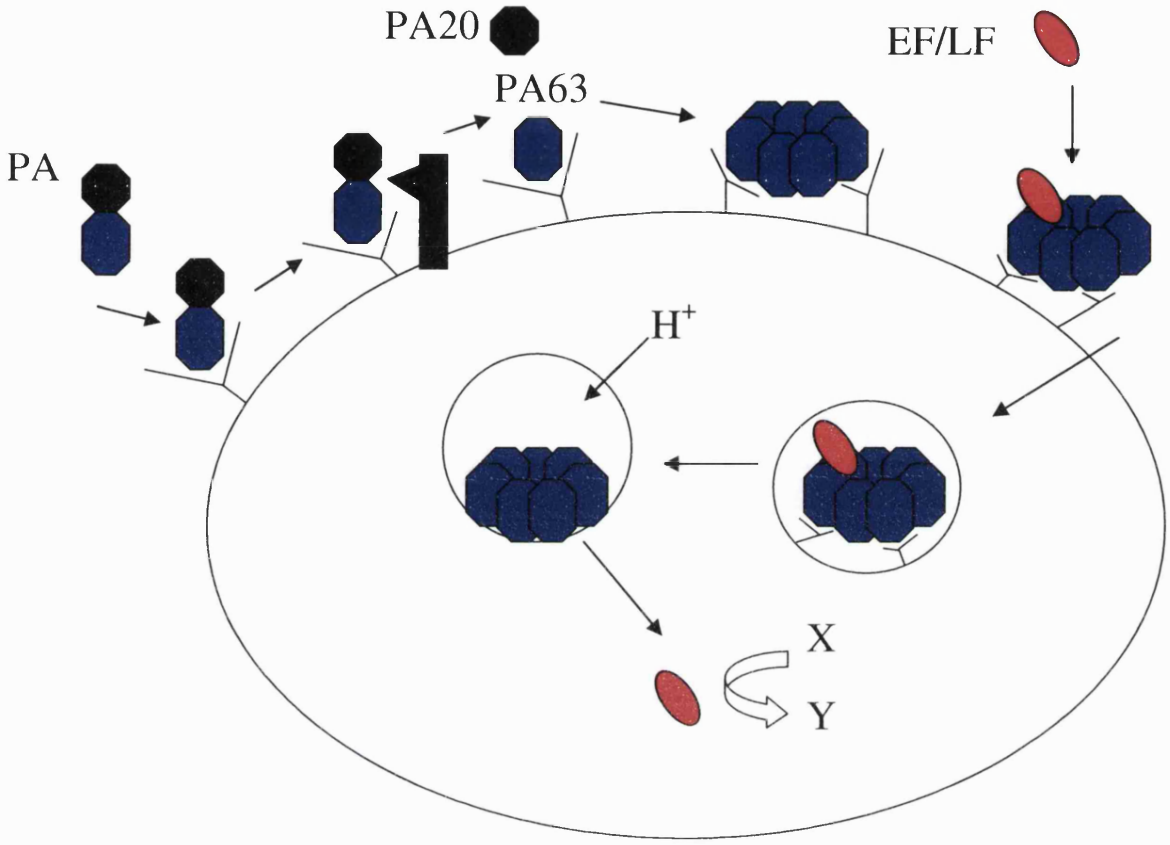


Figure 1.1 – Steps in anthrax intoxication. PA binds to the extracellular receptor and is activated by furin like proteases. PA20 then diffuses away whilst PA63 oligomerises to form a heptamer. EF and / or LF then bind to the heptamer and are internalised by receptor-mediated endocytosis. Acidification of the endosome then leads to pore-formation and the translocation of EF and / or LF to the cytosol where they can exert their cytotoxic effects (Adapted from Lacy and Collier 2002).

1.3.3.2 Oedema Factor (EF)

The EF gene, *cya*, is located on the pX01 plasmid and has been independently cloned and sequenced by two groups (Mock *et al.*, 1988; Robertson *et al.*, 1988). It contains a 2400 bp open reading frame of which 99 bp encode a hydrophobic 33 amino acid signal sequence and 2301 bp encoded the AT rich mature protein.

The mature EF protein is 767 residues with a molecular mass of 89 kDa (Robertson *et al.*, 1988). It is comprised of 2 functional domains. The N-terminal domain is responsible for the binding of PA and the C-terminal domain is a calmodulim dependant adenylate cyclase (Lacy *et al.*, 2002; Leppla, 1982; Little *et al.*, 1994) that is modulated by physiological calcium concentrations (Kumar *et al.*, 2002; Shen *et al.*, 2002). The catalytic domain shares three regions of homology with the catalytic region of the *Bordetella pertussis* adenylate cyclase enzyme, the only other known calmodulin dependant bacterial adenylate cyclase (Escuyer *et al.*, 1988). The first region of homology is believed to be the ATP binding motif and the remaining two regions are assumed to be involved in the stabilisation of calmodulin binding (Labruyère *et al.*, 1990; Munier *et al.*, 1993).

The characteristic oedema observed in cutaneous anthrax infections is due to the adenylate cyclase activities of EF. This oedema is a result of the production of intracellular cyclic AMP (cAMP) from host ATP (Leppla, 1982). Studies have shown that all cell types tested to date are sensitive to the EF and the magnitude of cAMP accumulation is dependant on cell type (Kumar *et al.*, 2002). The increase in cAMP can be up to 1000- fold, representing conversion of 20-50% of the intracellular store of ATP (Bhatnagar and Batra 2001; Gordon *et al.*, 1988). High doses of EF are not lethal since high levels of cAMP are not cytotoxic, however the accumulation of cAMP does induce the production of interleukin (IL) – 6 and inhibits the production of tumor necrosis factor (TNFα) in monocytes (Hoover *et al.*, 1994). The oedema toxin has also been shown to inhibit phagocytosis and oxidative burst of neutrophils (Confer and Eaton, 1982; Little and Ivins, 1999).

The importance of oedema toxin in terms of systemic anthrax is still under debate. Isogenic strains of *B. anthracis* that only express ET are attenuated 10 fold over wild type strains in mice (Pezard *et al.*, 1991, Pezard *et al* 1993), however co-administration of ET and LT does enhance the lethality of LT. Therefore ET may indirectly increase host susceptibility to infection by disrupting the cytokine response of monocytes and by suppressing neutrophil activities (Little and Ivins, 1999).

1.3.3.3 Lethal Factor (LF)

LF is encoded by *lef* (Bragg and Robertson, 1989; Robertson and Leppla, 1986) and the mature protein contains 776 residues and has a series of four imperfect repeats in the central part of the molecule that stabilise the protein (Quinn *et al.*, 1991). The structure of LF has been solved to 2.2Å, and has been shown to consist of 4 domains (Pannifer *et al.*, 2001). As with EF, the N-terminal (Domain I) is responsible for the binding to PA (Bragg and Robertson, 1989; Lacy *et al.*, 2002) and the C-terminal (Domain IV) forms the catalytic region of the protein. This catalytic region contains a thermolysin-like active site and a zinc-binding consensus motif, HExxH (Klimpel *et al.*, 1994). Mutations in this region abolish lethal toxin activity along with the binding of zinc, indicating that LF acts as a zinc metalloprotease (Hammond and Hanna, 1998). This is further supported by sequence analysis that shows that LF has a similarity to both thermolysin and leukotriene A_4 hydrolase and can therefore be inhibited by leukotriene A_4 hydrolase and metalloprotease inhibitors (Menard *et al.*, 1996).

Studies have shown that LF acts on a variety of substrates including peptide hormones granuliberin R, dynorphin A peptide, neurotensin, kinetensin and angiotensin-1 (Hammond and Hanna, 1998). However, the majority of studies to date have concentrated on the cleavage of the N-terminus of mitogen activated protein kinease kineases (MAPKKs). Currently 7 different MAPKKs have been characterised (Widmann *et al.*, 1999) and it is believed that LF cleaves all of these with the exception of MEK5 (Pellizzari *et al.*, 1999; Vitale *et al.*, 1998; Vitale *et al.*, 2000). This cleavage is known to occur at sites containing basic amino acids and prolines

(Chopra *et al.*, 2003; Duesbery *et al.*, 1998; Vitale *et al.*, 1998), however the complete cascade of events following the cleavage remains unclear. It is known that the MAPK pathway relays environmental signals to the transcriptional machinery in the nucleus, modulating gene expression (Brossier and Mock, 2001; Widmann *et al.*, 1999). In addition, MEK1, MEK2 and MKK3 have been shown to play a crucial role in the activation of macrophages and the production of the cytokines; TNF-α, IL-1 and IL-6 (Hoffmeyer *et al.*, 1999; Pellizzari *et al.*, 1999). Therefore, the release of these pro-inflammatory cytokines due to the actions of LF, may account for the shock seen in anthrax infections. Currently, however, there is conflicting evidence concerning the modulation of cytokines by LF. Reports have stated that sublytic concentrations of LT induce macrophages to express TNF-α and IL-1β (Hanna *et al.*, 1993), where as other data show that the release of TNF-α, IFN-regulatory factor 3 and NO are inhibited by LT (Dang *et al.*, 2004; Erwin *et al.*, 2001; Pellizzari *et al.*, 1999).

Further studies into the mechanism of action of LT have also suggested a role for the proteasome. This is believed to add to the toxic actions of LF by causing the degradation of molecules required for host cell homeostasis. Data show that proteasome inhibitors protected macrophages from LT but did not block the cleavage of MEK1, indicating that the proteasome is involved downstream of MEK1 (Tang and Leppla, 1999). In addition, it also been shown that protein synthesis, phospholipase C and protein kinase C are all required for LT cytotoxicity (Bhatnagar and Friedlander, 1994; Bhatnagar *et al.*, 1999).

It was initially believed that macrophages were uniquely susceptible to the actions of LT (Friedlander, 1986; Popov *et al.*, 2002); however LT has now also been reported to induce human endothelial cell apoptosis (Kirby, 2004) and to impair the adaptive immunity of dendritic cells (Agrawal *et al.*, 2003), inferring that LT actually has an influence on other cell types. Studies with macrophages have shown that incubation with LT leads to an increase in permeability, gross changes in cell morphology and cell lysis within 2 hours. Evidence also suggests that macrophages undergo both necrotic and apoptotic cell death in the presence of LT and that cell lysis maybe due

to the cell's own oxidative burst (Bhatnagar and Batra, 2001; Hanna *et al.*, 1992; Popov *et al.*, 2002). It has been reported that macrophages are a major producer of reactive oxygen species and hence, this may explain why they appear to be more sensitive to LT than other cell types (Friedlander, 1986). Susceptibility of mouse macrophages has also been shown to be determined by the gene *ltx1*, located on chromosome 11. This gene locus encodes Kif1C, a kinesin like motor protein of the UNC104 subfamily (Roberts *et al.*, 1998; Watters *et al.*, 2001). In addition, other studies have implied that TNF-α may also influence host cell resistance to LT, since it has been shown to induce components that co-operate with LT in inducing cell death in resistant macrophages (Kim *et al.*, 2003a).

1.3.3.4 Regulation of *B. anthracis* toxin genes

Studies have shown that the production of toxins in *B. anthracis* is regulated by *atxA,* the anthrax toxin activator. This gene was discovered by two groups independently (Koehler *et al.*, 1994; Uchida *et al.*, 1993b), it is 1.4 kb in size and is located on the pX01 plasmid. *atxA* has been shown to activate the translation of all three toxin genes, (Dai *et al.*, 1995; Uchida *et al.*, 1993b) since an *atxA* mutant has been shown to lack toxin production (Dai and Koehler, 1997). The mechanism by which *atxA* controls gene expression is not currently known, however the *atxA* gene has been shown to code for a 56 kDa protein, AtxA. This has been shown to share amino acid homology to AcpA (47%) and AcpB (50%), the capsule gene regulators (Drysdale *et al.*, 2004), and recent studies have shown that *atxA* may also have a role in cap gene regulation.

1.4 Anthrax vaccines

Anthrax vaccines have been designed and in use for a number of decades. Although they were initially produced in order to reduce the incidence of disease in livestock and their handlers, the current focus of vaccine design is the defence against bioterrorism and biological warfare.

There is only a limited amount of knowledge regarding human immunity to *B. anthracis* infections. It is believed that immunity to anthrax infection after a cutaneous infection is likely since re-infections are rarely reported and tend to be less severe than primary infections. In addition, animal data show that cases surviving infection are more resistant to subsequent challenges. There is also evidence showing that immunity can be transferred through serum of animals vaccinated with spores, culture filtrates and PA alone (Friedlander *et al.*, 2002; Little *et al.*, 1997), strongly suggesting that antibodies are the main mechanism of resistance and that immunity to infection can be induced by vaccination.

1.4.1 Live attenuated spore vaccines

The initial anthrax vaccines developed were live attenuated vaccines. The first one to be used was developed by Pasteur in 1881 and this was followed in 1939 with the Sterne vaccine. This latter vaccine has been shown to be effective and safe for use in domestic animals, despite showing low levels of virulence in guinea pigs and mice and is therefore still the veterinary vaccine of choice (Little and Ivins, 1999; Turnbull, 1991).

A live vaccine has also been developed in the former Soviet Union for use on humans. This was initially produced in the 1940s and was designed to protect humans from both naturally occurring disease and from the use of *B. anthracis* as a biological weapon. The vaccine was based on STI-1, a Sterne like *B. anthracis* strain and was administered by scarification of the shoulder with approximately 4 x 10^8 *B. anthracis* spores (Shlyakhov and Rubinstein, 1994; Turnbull, 1991).

1.4.2 Acellular vaccines

Due to the safety implications of using live spore vaccines, a number of chemical anthrax vaccines have also been developed. It has been shown that the presence of PA is both necessary and sufficient in inducing immunity (Ivins *et al.*, 1998; Little and Knudson, 1986; Pezard *et al.*, 1995), therefore both the current United Kingdom and United States vaccines are PA based (Friedlander *et al.*, 2002; Puziss and Wright, 1962; Turnbull, 1991).

The US anthrax vaccine adsorbed (AVA) was first licensed in 1970 and is an aluminium hydroxide adsorbed cell free filtrate of a non-capsulating non-proteolytic derivative of strain V770 from a case of bovine anthrax. The current U.K. vaccine has been licensed for over 40 years and consists of an alum-precipitated cell-free filtrate, produced from culture supernatants of the Sterne non-capsulating strain of *B. anthracis* (Friedlander *et al.*, 2002; Turnbull, 1991). The kinetics of production of PA and the other toxins in these methods are poorly understood and for this reason the resulting batches show variations (Leppla *et al.*, 2002; Personal communication – Richard Sharp, HPA). The UK vaccine has, however, been shown to produce greater levels of EF and LF than the US vaccine (Turnbull *et al.*, 1986; Turnbull *et al.*, 1988).

These vaccines are currently recommended for individuals at an occupational risk, specifically those who work with animal hides, wool and bones, as well as laboratory workers. More recently the anthrax vaccines have also been administered to members of the armed forces. With the recent bio-terrorist attack of anthrax in the USA, the vaccine has also been given to pre-exposed individuals, however the overall benefit of this treatment is still unclear (US Department of Health and Human Services). Unfortunately there is a lack of human efficacy data for either of the vaccines, since at the time that the human vaccines were introduced, cases were on the decline due to improved factory hygiene and monitoring. A study was completed in the US, comparing mills that had and had not received the vaccine – this indicated 92.5% effectiveness with the US vaccine (Turnbull, 1991). Tests on animals however have indicated that the effectiveness of the vaccines are less than ideal (Ivins *et al.*, 1986a;

Little and Knudson, 1986; Turnbull *et al.*, 1988; Turnbull, 1991). A number of immunity studies have been completed with these vaccines and have demonstrated that although anti-PA responses are frequently high, no correlation is present between anti-PA titres and levels of protection. Correlation is however, present between neutralising antibody titres and protection, suggesting that neutralising antibodies are a key component of protective immunity and may act as a marker for protection (Little and Ivins, 1999; Reuveny *et al.*, 2001).

Unfortunately, immunisation with both the UK and US human anthrax vaccines can induce local pain and erythemas. Both vaccines also have complex immunisation schedules with frequent boosters required (Bhatnagar and Batra, 2001). This therefore leaves scope for improvement and development of new vaccines.

1.4.3 Future vaccines

Numerous projects are currently attempting to produce an improved human vaccine for use against *B. anthracis*. These vaccines have the ultimate aim of being safe, non-reactogenic, suitable for civilian populations, effective against all forms of anthrax infection, be easy to administer, use a minimal number of immunisations and achieve immediate and long lasting immunity. The vaccines must also be readily producible on a large scale.

1.4.3.1 PA vaccines

In the medium term it is believed that PA subunit vaccines are the only candidates likely to receive licensing approval. Therefore, numerous attempts have been made to express PA at high levels in a variety of organisms including attenuated *B. anthracis* (Farchaus *et al.*, 1998; Turnbull, 1991), *B. subtilis* (Baillie *et al.*, 1994; Baillie *et al.*, 1998; Ivins and Welkos, 1986b; Miller *et al.*, 1998), *B. brevis* (Baillie, 2001), *Vaccinia* and *Baculovirus* (Iacono-Connors *et al.*, 1990) *Salmonella typhimurium* (Coulson *et al.*, 1994) and *E. coli* (Chauhan *et al.*, 2001; Sharma *et al.*, 1996; Vodkin and Leppla, 1983), with the best reported yields seen in the *Bacillus* strains. The plasmid encoded PA gene is expressed well in *B. subtilis* and achieves higher levels

of expression than that seen in the current *B. anthracis* system (Ivins and Welkos, 1986b); however the drawback from using bacilli is their ability to produce proteases that hamper purification of recombinant PA. rPA levels have however been increased in a protease deficient *B. subtilis* strain and studies have shown that the recombinant PA protein is able to protect a variety of animals against an anthrax challenge (Miller *et al.*, 1998).

Further to these results, in October 2002 the National Institute of Allergy and Infectious Disease (US) awarded grants to Vaxgen (US) and Avecia (UK) to initiate phase I clinical trials to develop, test, manufacture and stockpile new recombinant PA anthrax vaccines. In addition, in September 2003 Vaxgen was awarded a further grant to enter phase II clinical trials and more recently, in November 2004, received a further award from the US government to produce 75 million doses of the rPA102 vaccine and to enter phase III clinical trails. These contracts therefore clearly show the potential for recombinant PA vaccines to be the basis of the next generation of anthrax vaccines (www2.niaid.nih.gov; www.vaxgen.com).

1.4.3.2 Adjuvants

A number of studies have also been completed using PA combined with a variety of adjuvants. Aluminium salts are currently one of the few adjuvants approved for human vaccination and are believed to be potent stimulators of humoral rather than cellular immunity (Jendrek *et al.*, 2003). Numerous experimental PA adjuvants have been tested in the US and the UK and the ability of some of these PA / adjuvant combinations to prevent disease has been demonstrated in studies on guinea pigs (Ivins *et al.*, 1995; Miller *et al.*, 1998; Singh *et al.*, 1998). The findings from these studies show variable results. Many of the candidate adjuvants do not elicit greater protection or serological response than the current licensed vaccines; however some of the candidate vaccines, including Ribi (Miller *et al.*, 1998) saponin QS-21 and monophosphoryl lipid A (Ivins *et al.*, 1995) show a high level of protection to guinea pigs against spore challenge.

A further line of research is the use of synthetic carrier systems for the delivery of PA. Micro-encapsulation of antigens has a number of advantages over the current UK and US vaccines, since it does not require refrigeration and has the potential for using less intrusive methods of delivery. Studies have been completed investigating the use of poly-L-lactide microspheres and polyactide-co-glycolide (PLG) microparticles for PA and AVA respectively. Studies encapsulating PA in poly-L-lactide microspheres or attaching rPA to microspheres by lyophilization have shown that vaccination of guinea pigs, either by the intramuscular or intranasal routes, leads to protection from an injected challenge of 10^3 median lethal does of *B. anthracis* STI spores as well as leading to protection against aerosol challenge with 30 median lethal doses of STI spores (Flick-Smith *et al.*, 2002).

Studies have previously shown that synthetic oligonucleotides containing immuno-stimulatory CpG motifs (CpG OLN) can boost the immune response to co-administered antigens including AVA (Klinman *et al.*, 2004). Further studies have combined CpG OLN with PLG, these studies have shown that the use of CpG OLN-PLG with AVA leads to a stronger and faster IgG response against PA than AVA alone (Xie *et al.*, 2005). These findings therefore indicate that, in addition to the development of new adjuvants, the use of synthetic carriers may also lead to an improved human anthrax vaccine.

1.4.3.3 PA mutants

The gene encoding PA has also been mutated to generate a non-cleavable PA mutant. Studies using the mutated PA alone or in combination with EF and LF show high anti-PA titres and protected guinea pigs against spore challenge (Singh *et al.*, 1998). Other deletion mutants in PA that block pore formation and translocation have also been generated (Sellman *et al.*, 2001b). These have also been found to inhibit the actions of anthrax toxins *in vivo,* protecting mice against toxin challenge (Ahuja *et al.*, 2003). These results show that mutants in PA may be another possible route in generating effective recombinant vaccines.

1.4.3.4 Spore vaccines

Despite progress in new acellular vaccines, immunisation studies that have compared live spore vaccines with chemical vaccines have shown that the live vaccines afford greater protection in guinea pigs although the chemical vaccines produce greater anti-PA titres (Little and Knudson, 1986). In addition, spore vaccines have also been shown to be more effective than vegetative cell vaccines. This therefore indicates that either spore antigens contribute to protection, or their physical presence may enhance efficacy by targeting the antigens to immune cells and enhancing phagocytosis of the vaccine (Brossier *et al.*, 2002; Cohen *et al.*, 2000; Oggioni *et al.*, 2003; Pezard *et al.*, 1995; Stepanov *et al.*, 1996). This is further emphasised by a study that compared immunisation with PA alone and PA in combination with formaldehyde-inactivated spores. The combination of spores and PA gave 100% protection, where as PA alone only afforded 50% protection in a mouse challenge, thus indicating that the addition of spores enhanced the immunisation efficiency (Brossier *et al.*, 2002).

Other research has generated a non-toxigenic and non-encapsulated recombinant spore vaccine that expresses high levels of recombinant PA. This induced high levels of neutralising antibodies and protected guinea pigs against challenge. The recombinant spore vaccine has also been reported to be more efficacious than the vegetative cell vaccine (Cohen *et al.*, 2000).

1.4.3.5 Recombinant live vaccines

Since spores have been implicated in enhancing protection, studies have also been completed on the generation of recombinant live vaccines. Recombinant DNA technology methods have been used to generate live vaccine strains of *B. anthracis* (Barnard and Friedlander, 1999; Cohen *et al.*, 2000), *B. subtilis* (Ivins and Welkos, 1986b), *Salmonella* (Coulson *et al.*, 1994; Garmory *et al.*, 2003;) and *Vaccinia virus* (Bennett *et al.*, 1999) that produce PA but not EF or LF. These vaccines only require a single dose by oral administration, since following delivery the live bacteria colonises the mucosal surface and causes a limited infection during which the vaccine candidate is expressed and presented to the immune system. These vaccines are,

however disadvantaged since the live organisms need to be cultured and stored prior to use (Baillie, 2001).

1.4.3.6 Mutant vaccines

In addition, aromatic compound dependant mutants of toxigenic non-encapsulated *B. anthracis* strains have also been developed. These mutants have been shown to be attenuated in mice and guinea pigs and showed protection against a highly virulent Ames challenge in these animal models. Unfortunately, their development as potential live vaccine candidates is hindered by the requirement for the elimination of the self-transmitting tetracycline resistance factor. Despite this, the idea of mutations in the aromatic acid pathway is still believed to be of potential therapeutic use (Ivins *et al.*, 1990).

Other studies have involved the development of a vaccine based on a *B. anthracis* mutant inactive in both metalloprotease and adenylate cyclase activity. Although administration of this mutant has not been compared with the Sterne vaccine or demonstrated in an animal model closely resembling human anthrax, it has been shown to protect mice against a virulent challenge of *B. anthracis*. It has therefore been suggested to have potential as live vaccine candidate, since it is fully attenuated whilst processing a number of the other immunogens possessed by Sterne (Brossier *et al.*, 2000; Friedlander *et al.*, 2002).

1.4.3.7 Genetic vaccination

A further line of research has been the generation of DNA vaccines. These have been shown to be extremely simple and cost effective and are broadly defined as DNA expression vectors. Administration of these vaccines results in the expression of an antigen *in situ* leading to the induction of antigen specific immunity. The DNA vaccines have the advantage of being simple to produce but have numerous problems in eliciting effective delivery of plasmid DNA. Recent studies have shown that intramuscular vaccination with DNA encoding PA induced toxin neutralising antibodies and protected mice from low doses of lethal toxin (Gu *et al.*, 1999),

however a similar DNA plasmid encoding PA has also been tested on guinea pigs. In this study four doses of DNA delivered via a gene gun induced low levels of antibodies, however failed to protect the animals against an intramuscular challenge with a virulent strain of *B. anthracis* (Friedlander *et al.*, 2002). It can therefore be seen that although DNA vaccine technology can be used as a potential method for prevention of anthrax infection, further work on effective delivery mechanisms are still required to develop a protective vaccine against spore challenge.

1.4.3.8 EF and LF

Since the UK and US vaccines are prepared from culture supernatants they contain a number of bacterial derived proteins as well as PA, these include EF and LF. Since these proteins may influence the immune response to the anthrax vaccine, studies have also been completed on their roles in the vaccines. Unfortunately, work in this area has led to a number of contradictory results and therefore the true benefits of adding these antigens to future vaccines remains unclear.

Initial work in this area indicated that LF and EF alone are only capable of inducing relatively low levels of protection and hence their addition has no therapeutic benefit in anthrax vaccines (Ivins and Welkos, 1988; Little and Knudson, 1986). However more recent studies have reported that a strain producing LF alone can provide equivalent protection as a strain producing only PA and strains producing combinations of PA and LF or PA and EF are more protective than PA alone, indicating that EF and LF have an adjuvant effect on of PA (Pezard *et al.*, 1995). Furthermore, it has also been reported that PA negative mutants are capable of providing a degree of protection against anthrax infections (Friedlander *et al.*, 2002), although pX01 mutants are not. This therefore indicates that LF, EF or some other pX01 encoded non-toxin antigen contributes to protection. These data therefore infer that there is a benefit to adding EF, LF and other non-toxin bacterial derived components to anthrax vaccines. However, since these components may also add to the undesired side effects seen with the present vaccine, further work is required to

fully understand if these factors have an overall positive contribution to anthrax vaccines.

1.4.3.9 Capsule and S-layer proteins

The capsule alone has been shown to be a poor immunogen and therefore few studies have been completed on its protective effects (Chabot *et al.*, 2004; Schneerson *et al.*, 2003). The reports available have shown that capsule vaccines may enhance protection against *B. anthracis*. Studies administering capsule alone have shown that it is protective against a lethal challenge with a capsulated non-toxigenic *B. anthracis* strain. In addition, the capsule has also been shown to enhance the protection of mice challenged with a fully virulent strain when administered with PA (Chabot *et al.*, 2004). Furthermore, other studies have developed a dually active vaccine that is capable of protecting mice against both bacilli and toxins. This vaccine is based on the conjugation of PA to poly-γ-D-glutamic acid (PGA), allowing the generation of both anti-PA antibodies, neutralising toxin activity and anti-PGA antibodies, promoting the killing of encapsulated bacilli (Rhie *et al.*, 2003; Schneerson *et al.*, 2003).

It has also been shown that when *B. anthracis* does not produce a capsule, the exterior cell wall appears layered due to the S-layer. This layer is comprised of two proteins; extractable antigen 1 (EA1) and surface array protein (Sap) (Etienne-Toumelin *et al.*, 1995; Mesnage *et al.*, 1997). These proteins have been demonstrated to be immunogenic in animals and man, although immunisation with them alone does not afford protection (Baillie *et al.*, 2003). It is however possible that the S-layer proteins, in combination with PA may enhance protection by causing the production of further antibodies directed against the bacterium and hence still may be potent vaccine components (Baillie *et al.*, 2003; Mesnage *et al.*, 1997).

1.4.4 Vaccine Summary

Overall, it can be seen that whilst human and animal vaccines are currently available for immunisation against *B. anthracis*, several other developments are underway in order to improve the current vaccines. These developments have been accelerated in recent years due to the increased threat of bioterrorism and biological warfare. The current major aim of the improved vaccines are for them to be potent and both simple and rapid to administer. Unfortunately, at present no vaccine is currently available that meets all of these requirements and hence multiple lines of research are being studied in order to develop an appropriate vaccine.

1.5 *Bacillus cereus*

Due to its close relationship to *B. anthracis* (as described in section 1.2.1) and the restrictions imposed when studying a category 3 organism, other *Bacillus* species, including *B. cereus* are often used as a surrogates for *B. anthracis* in microbiological research (Beuchat *et al.*, 2004; Nicholson and Galeano, 2003; Utrup *et al.*, 2003; Weber *et al.*, 2003). Nevertheless, *B. cereus* is in itself a category 2 organism and has also been shown to be pathogenic.

1.5.1 Pathogenesis

B. cereus has a worldwide distribution and is commonly found in the soil. It has been described as an opportunistic pathogen and has been located from various infections in humans. It is a food-borne pathogen and is often found in cooked rice, pasta and meat, eggs and other dairy products (Kotiranta *et al.*, 2000). *B. cereus* causes numerous problems in food production and processing since it is a ubiquitous microbe in the environment and can survive inadequate pasteurisation and heating. The spores are also reasonably resistant to gamma-ray irradiation, a common treatment used to reduce food pathogens and are able to adhere to surfaces causing further problems in dairy industries (Andersson *et al.*, 1998; Larsen and Jørgensen, 1999; Rönner *et al.*, 1990; Stalheim and Granum, 2001).

1.5.1.1 Food poisoning

B. cereus is responsible for two forms of food poisoning; the diarrhoeal type caused by a complex of enterotoxins and the emetic-type syndrome. Exact numbers of cases are unknown since *B. cereus* infections are not reportable and are not always diagnosed (Kotiranta *et al.*, 2000). Both types of poisoning often occur following consumption of re-heated cooked foods, where endospores have been allowed to germinate. The infective dose seems to vary between 10^5 - 10^8 spores (Granum and Lund, 1997). Emetic food poisoning has commonly been associated with rice and pasta. It has a rapid onset between 1-5 hours and symptoms include nausea and vomiting. These symptoms normally resolve after 24 hours. The diarrhoeal type is transmitted mainly by milk products, vegetables and meats. After an 8-16 hour

incubation time, patients develop symptoms of abdominal pain and cramps, along with diarrhoea. These symptoms normally last between 12-24 hours (Granum, 1994; Kotiranta *et al.*, 2000).

1.5.1.2 Non gastrointestinal infections

Non gastrointestinal infections have also been reported for *B. cereus*, especially in immuno-compromised patients, neonates, drug addicts and patients with traumatic wounds, surgical wounds or catheters. It is likely that the production of the enterotoxins and emetic toxin are important in the establishment of these infections (Beecher *et al.*, 1995a; Drobniewski, 1993; Sliman *et al.*, 1987; Turnbull *et al.*, 1979; Turnbull and Kramer, 1983).

Local *B. cereus* infections tend to manifest as post-surgical or traumatic wounds, burns and osteomyelitis. Although the majority of infections are mild, severe deep infections can also occur such as necrotising fasiitis and gangrene. *B. cereus* has also been reported to be one of the most important organisms found in severe ocular infections, including keratitis, endophthalmitis and panophthalmitis (Drobniewski, 1993; Kotiranta *et al.*, 2000; Turnbull *et al.*, 1979).

Systemic *B. cereus* infections are extremely rare, since the bacteria are normally transient and harmless. However *B. cereus* has been reported to cause a number of severe infections including *B. cereus* pneumonia, urinary tract infection, root canal infection, endocarditis, meningitis, encephalitis and fatal liver failure (Barrie *et al.*, 1992; Berner *et al.*, 1997; Drobniewski, 1993; Kotiranta *et al.*, 2000; Mahler *et al.*, 1997; Marley *et al.*, 1995; Sato *et al.*, 1998; Steen *et al.*, 1992).

1.5.2 Proteases

B. cereus infections are caused by a number of virulence factors encoded on the *B. cereus* genome. One of these factors is the ability to produce a range of proteases. Unfortunately, at present very few reports currently exist on *B. cereus* proteases and

their role in virulence; however it is believed that they play a role in non-gastrointestinal *B. cereus* infections (Kotiranta *et al.*, 2000).

Extracellular proteases have been detected and characterised from a number of *B. cereus* strains including *B. cereus* KCTC 3674 and *B. cereus* BG1. Stability and inhibition tests on these proteases have shown them to be metalloproteases (Ghorbel *et al.*, 2003; Kim *et al.*, 2003b). In addition, a neutral protease from *B. cereus* has also been sequenced and found to have homology to a metalloprotease from *B. thuringiensis.* This protease has been reported to be a thermolysin-like enzyme (Donovan *et al.*, 1997) and further studies have found it to be highly conserved during the evolution of the *B. cereus* group (Bach *et al.*, 1999). A further neutral protease from *B. cereus* has also been shown to have a damaging effect on haemoglobin and albumin, (Sierecka, 1998) supporting reports that *B. cereus* proteases are virulence factors in non-gastrointestinal diseases.

In addition to the extracellular proteases produced by *B. cereus*, a cell-envelope bound metalloprotease, camelysin, has also been implicated as a possible pathogenic factor. This metalloprotease has been characterised as a neutral zinc metalloprotease, with a molecular mass of 19 kDa, encoded by the *calY* gene (Fricke *et al.*, 1995; Fricke *et al.*, 2001; Grass *et al.*, 2004). Disruption of the *calY* gene results in a decrease in cell bound proteolytic activity. Camelysin has been shown to have a broad specificity, cleaving a variety of substrates, including casein and has been shown to be expressed on the surface of vegetative cells during the logarithmic growth phase, when grown in complex media (Grass *et al.*, 2004). It has also been shown to interact with proteins in the blood coagulation cascade and hence may facilitate the prevention of fibrin clots and extracellular matrix during bacterial infection (Fricke *et al.*, 2001).

1.5.3 Emetic toxin

The *B. cereus* emetic toxin, cereulide, is an extremely stable, cyclic dodecadepsipeptide, that is resistant to proteolytic degradation, pH extremes and heat treatment, (Granum and Lund, 1997) since it is capable of surviving at 126°C for 90

minutes (Melling and Capel, 1978). The structure has a molecular mass of 1.2 kDa and resembles the potassium ionophore antibiotic valinomycin that has been shown to have similar biological activities, albeit at higher doses (Agata *et al.*, 1994).

1.5.3.1 Mechanism

The biological mechanism by which cereulide acts is still not fully understood, however it has been shown to stimulate the vagus afferent through the 5-HT3 receptor and has been shown to cause swelling in the mitochondria of Hep-2 cells (Agata *et al.*, 1995a). Research has also shown that the emetic toxin is capable of inhibiting the activity of the human natural killer (NK) cells, by inhibiting cytotoxicity and cytokine production. The emetic toxin has also been shown to cause swelling of NK mitochondria and eventually induces NK cell apoptosis. The effect on NK cytotoxicity has been shown to be fast and can occur at low concentrations (Paananen *et al.*, 2002).

1.5.3.2 Detection

The emetic toxin is non antigenic, hence its detection by immunological methods has proved difficult. Earlier detection methods were either *in vivo* primate challenges (Agata *et al.*, 1994) or *in vitro* based on Hep-2 cell assays. When cereulide is added to the HEp-2 cells there is a change in the vacuoles along with a change in acid production, cell rounding, cell granulation and cytostatic activity (Szabo *et al.*, 1991). The colour of metabolites was also found to be different when cells were inhibited by the emetic toxin, providing the basis of one emetic toxin assay (Mikami *et al.*, 1994), with a second Hep2 assay was based on vacuole formation (Hughes *et al.*, 1988). More recent assays have been the sensitive, semi-quantitative, semi-automated MTT dye based assay for the emetic toxin (Finlay *et al.*, 1999) and the boar sperm motility assay, since boar sperm motility is inhibited in the presence of cereulide via mitrocondiral damage (Jääskeläinen *et al.*, 2003).

1.5.4 Enterotoxins

In addition to the formation of the emetic toxin, *B. cereus* also produces a number of enterotoxins that are responsible for the diarrhoeal food poisoning syndrome. The number of enterotoxins and their properties are under debate (Granum and Lund, 1997), however at least five enterotoxins have currently been described, three of which are known to cause food poisoning syndromes.

1.5.4.1 Mechanism

The action of the enterotoxins at the molecular level is still not fully understood. However the toxins are known to reverse the adsorption of fluid, Na^+, and Cl^-, and cause malabsorption of glucose and amino acids. The toxins are also capable of causing neurosis and mucosal damage (Granum *et al.*, 1993a; Mäntynen and Lindström, 1998). The preformed toxin has been shown to be unable to survive ingestion and therefore enterotoxin poisoning is thought to be through the ingestion of spores that then germinate and produce the toxins in the intestine (Drobniewski, 1993; Granum *et al.*, 1993a; Lund, 1990; Shinagawa *et al.*, 1991).

1.5.4.2 Haemolysin BL

The main focus of *B. cereus* enterotoxin study has been on Haemolysin BL (HBL). This is a three component enterotoxin, comprised of a B, binding subunit that is 37.5 kDa and two lytic components; L_1, 38.2 kDa and L_2, 43.5 kDa. The pI values of the components are also very similar at 5.34, 5.33 and 5.33 respectively. Maximal enterotoxic activity requires all three subunits and none of the individual components have exhibited any toxic activity alone, however combinations of the B subunit with either L_1 or L_2 have been shown to have low levels of haemolytic activity (Beecher and Wong, 1994a; Beecher *et al.*, 1995b).

1.5.4.2.1 Genomic arrangement

The genes encoding the B, L_1 and L_2 components, *hblA*, *hblD* and *hblC,* respectively, have been cloned and sequenced (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997), and are represented in Figure 1.2. Data show that they are transcribed from the same operon

and have a 5.5 kb RNA transcript with a promoter sequence 608 bp upstream (Lindbäck *et al.*, 1999; Ryan *et al.*, 1997). The operon has been mapped to the unstable part of the *B .cereus* chromosome (Carlson *et al.*, 1996). A gene inactivation mutant has been created in the *hblC* gene of *B. cereus* ATCC 14579. Studies using this mutant have shown that a disruption in the *hblC* gene causes a vast reduction in enterotoxigenic activity and haemolytic activity in sheep, but not human, erythrocytes (Lindbäck *et al.*, 1999). A fourth gene, *hblB*, is also present in the *hbl* operon in some strains. This is believed to encode the putative B' protein, that is very similar in the first 158 amino acids to the B protein. The length of the *hblB* gene is not known and neither is the function of the B' protein, however it is possible that it may be capable of acting as a substitute for the B protein (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997).

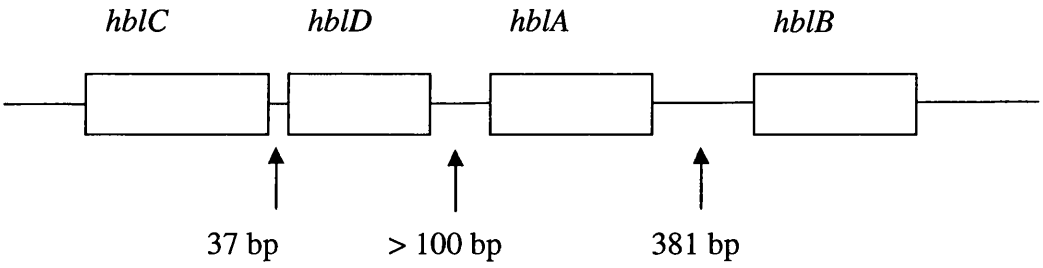


Figure 1.2 –The map of the HBL operon, that encodes for the HBL proteins; L_2 protein (*hblC*), L_1 protein (*hblD*), B' protein (*hblA*) and B protein (*hblB*) (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997).

1.5.4.2.2 Mechanism of action

Studies with HBL have shown that is able to cause a variety of toxic activities including cytotoxicity, vascular permeability, dermonecrosis, enterotoxicity and ocular toxicity (Beecher and Macmillan, 1991; Beecher and Wong, 1994a; Beecher *et al.*, 1995a; Beecher *et al.*, 1995b). In addition, it has also been shown to produce an unusual discontinuous hemolysis pattern on blood agar plates, with lysis beginning several millimetres away from the source (Beecher and Wong, 1997). Early studies into the mechanism of HBL indicated that the toxin acted by stimulating the cAMP

response in intestinal cells and subsequently caused fluid accumulation (Turnbull, 1976), although more recent studies have led to an alternative model. Studies using spectrophotometric analysis and immunofluorescent staining of erythrocytes have shown that the B component of HBL binds to susceptible cells, priming them to lysis by the L components. Studies have also revealed that at high concentrations of the B subunit, there is inhibition of L_1 mediated lysis and at high concentrations of the L components there is inhibition of the B component priming, explaining the discontinuous hemolysis patterns (Beecher and Wong, 1997). In addition, further studies have also revealed that B component induced priming of erythrocytes is a temperature sensitive event and the binding events of the HBL components are independent (Beecher and Macmillan, 1991; Beecher and Wong, 1994a; Beecher and Wong, 1997).

Furthermore, complete neutralisation of membrane bound L components by specific antibodies suggest that HBL causes hemolysis via the interaction of at least the L subunits with the erythrocyte membrane in 'a membrane attacking complex' and not by enzymatic degradation of the membrane. In addition, osmotic protection studies also indicate that this membrane attacking lysis occurs by a colloid osmotic lysis mechanism through trans-membrane pores (Beecher and Wong, 1997).

Other studies investigating the mechanism of HBL action have also reported that HBL may act on target membranes in conjunction phospholipases secreted by *B. cereus* (Drobniewski, 1993). It has also been suggested that the B and L components of HBL may be sphinomyelinases that are not haemolytic alone (Granum and Nissen, 1993).

1.5.4.2.3 Role in disease

HBL has been proposed as an important virulence factor in diarrhoeal food poisoning syndromes, however its exact role is still undefined (Beecher *et al.*, 1995b). It is likely that the enterotoxigenic properties of *B. cereus* results from a combination of toxic complexes, although the potency of the HBL toxin has been compared to that of

cholera toxin and has therefore been suggested to be the primary virulence factor of *B. cereus* (Beecher *et al.*, 1995b; Finkelstein and Lospalluto, 1972). Screening of a number of *Bacillus* strains has shown that the HBL toxin is broadly disrupted in the *B. cereus* group, causing further implications in food safety (Pruβ *et al.*, 1999). HBL has been shown to be lethal to mice, cytotoxic to Chinese hamster ovary cells and positive in the ileal loop test and vascular permeability reaction (Beecher and Wong, 1994a; Beecher *et al.*, 1995b).

1.5.4.2.4 Endophthalmitis

HBL has also been indicated as a major virulence factor in necrotising endophthalmitis, a highly virulent intra-ocular infection resulting from the introduction of *B. cereus* into the posterior segment of the eye (Beecher *et al.*, 1995a). The infection commonly results in the loss of the eye within 48 hours of infection despite the use of antibiotics and many reports have attributed this to toxin production (Beecher *et al.*, 1995a; Davey and Tauber, 1987; Vahey and Flynn, 1991). Research has shown that purified HBL toxin preparations are toxic to retinal cells and cause intraocular inflammation resembling *B. cereus* endophthalmitis. It has also been reported that HBL only accounts for half of the retinal toxicity of *B. cereus* supernatants suggesting that full virulence is a multi-component process (Beecher *et al.*, 1995a) and more recent data show that both phospholipase C and collagenase also contribute to the virulence of *B. cereus* in endophthalmitis (Beecher *et al.*, 2000a).

1.5.4.2.5 Toxin detection

A reverse passive latex agglutination assay has been developed to detect HBL and is commercially available (Oxoid RPLA). This assay detects the L_2 component of HBL through the use of polyclonal sera (Beecher and Wong, 1994b; Granum *et al.*, 1993b). More recently an improved cytotoxicity assay for *B. cereus* enterotoxin has been developed and compared to the Oxoid assay. This assay is an improved McCoy cell cytotoxicity assay and is claimed to be more sensitive than the Oxoid kit, although it does not distinguish between the cytotoxic effects from the various enterotoxins

(Fletcher and Logan, 1999). A further in vitro assay for the detection of HBL from *B. cereus* has also been established based on the hemolysis pattern on blood agar plates, whilst this assay is effective in demonstrating which strains are capable of producing HBL, it does not allow for quantification on the amount of HBL present (Fermanian and Wong, 2000). A MTT base assay has also been developed for the detection of *B. cereus* cytotoxicity, this assay is not dependant on subjective assessment of cell damage and enables quantitative measurement of toxicity in culture supernatants. The assay can be used to detect all of the enterotoxins along with the emetic toxin, however is not capable of measuring HBL levels alone (Beattie and Williams, 1999).

1.5.4.3 Non-haemolytic enterotoxin

The second most studied *B. cereus* enterotoxin is the non-haemolytic enterotoxin, Nhe. This was first isolated from a *B. cereus* strain associated with a food poisoning outbreak in Norway in 1995. It is composed of three subunits all of which are required for maximum cytotoxicity, although binary combinations have also been shown to exhibit some activity. Neither the complete complex nor any combinations of proteins have been shown to be haemolytic (Lund and Granum, 1996). Although the exact function of each of the individual proteins is unknown, it is believed that the 105 kDa subunit is responsible for binding Nhe to the target cells and variations in Nhe cytotoxicity maybe explained by variations in the 105 kDa protein (Lund and Granum, 1997). The 105 kDa protein has also been reported to be a metalloprotease with gelatinolytic and collagenolytic activities and studies have shown that it can be inhibited by EDTA and 1,10 phenanthroline (Lund and Granum, 1999).

1.5.4.4 Enterotoxin entFM

The entFM toxin is 45 kDA in size and has been purified and characterised. Few studies have been completed on this enterotoxin, but initial data show it to have both cytotoxicity and haemolytic activity (Luxananil *et al.*, 2003), contributing to *B. cereus* virulence.

1.5.4.5 Cytotoxin CytK

The cytotoxin, CytK, has been isolated from a *B. cereus* strain that caused food poisoning in an outbreak responsible for killing 3 people. The protein is highly cytotoxic as well as being both necrotic and haemolytic (Lund *et al.*, 2000). It has the ability to form pores in lipid bilayers and form oligomers that are resistant to sodium dodecyl sulphate, but not to boiling, in a similar way to other β-barrel pore-forming toxins (Hardy *et al.*, 2001). The amino acid sequence of the protein has also been shown to have a 30% similarity to a family of β-barrel channel forming toxins and therefore, CytK is thought to be responsible for a disease that is similar, but less severe, to the necrotic enteritis caused by the β-toxin of *C. perfringens* type C (Lund *et al.*, 2000).

1.5.4.6 *B. cereus* enterotoxin T

The *B. cereus* enterotoxin T, bceT, has also been described. This was originally believed to be one of the enterotoxic proteins in *B. cereus* responsible for food-borne diarrhoea (Agata *et al.*, 1995b), however, more recent reports have stated that it probably can not contribute to food poisoning (Choma and Granum, 2002), although may still have some role in *B. cereus* virulence.

1.5.5 Exotoxins

As well as production of proteases, emetic toxin and the enterotoxins, *B. cereus* also produces a number of other virulence factors in the form of exotoxins and membrane degrading enzymes. These virulence factors include at least three haemolysins; cereolysin and haemolysins II and III, as well as three phospholipases C (PLC); sphinomyelinase (SMase), phosphatidylcholine (PC) and phosphatidylinositol (PI).

1.5.5.1 Haemolysins

Haemolysins have a wide range of effects on various tissues and have a cytolytic action on mammalian cells. In addition, cereolysin has also been reported to have sub-lytic effects on leukocyte and macrophage actions (Drobniewski, 1993). Data suggest that the hemolysins belong to a group of β-channel forming cytolysins (Baida *et al.*,

1999). Studies have shown that hemolysin II is capable of forming a homoheptameric pore, as well as being a structural and functional homolog of *Staphylococcus aureus* alpha-toxin (Miles *et al.*, 2002).

The cytolysins are inactivated by cholesterol, the assumed membrane receptor and are irreversibly inhibited by oxidation. The mechanism of action for cereolysin and the other hemolysins is unclear, however it has been proposed that binding to the cellular receptor allows pore formation, through which intracellular leakage occurs leading to cell lysis (Drobniewski, 1993).

1.5.5.2 Phospholipase C

Phospholipase C (PC-PLC) is a monomeric metalloenzyme containing three Zn^{2+} in its active site (Hansen *et al.*, 1993; Hough *et al.*, 1989). It is encoded by the *plc* gene, that has been cloned, sequenced and expressed in *E. coli* (Johansen *et al.*, 1988; Tan *et al.*, 1997). Incubation with PC-PLC has been shown to result in the activation of the arachidonic acid pathway in a number of different mammalian cell types and also causes interference with protein kinase C regulation in oncogene-transformed fibroblasts (Diaz-Laviada *et al.*, 1990; Levine *et al.*, 1987). PC-PLC also leads to both activation and inactivation of neutrophils in various cell types (Styrt *et al.*, 1989; Wazny *et al.*, 1990) and has been shown to cause matrix metalloprotease up regulation in epithelial cells. These data therefore imply that PC-PLC may be involved in mediating tissue damage during infection (Firth *et al.*, 1997).

1.5.5.3 Sphingomyelinase

Sphingomyelinase (SMase) of *B. cereus* has also been cloned and sequenced (Yamada *et al.*, 1988). Functional studies have shown that it is able to adsorb onto erythrocyte membranes prior to lysis and is stimulated by Mg^{2+} but inhibited by Ca^{2+} (Ikezawa *et al.*, 1986; Tomita *et al.*, 1983). It is also able to induce apoptosis in several mammalian cell types through the generation of intracellular ceramide via the sphinomyelin pathway (Flores *et al.*, 1998). In addition, it has been reported that secretion of SMase is repressed by inorganic phosphate (Pi) in the growth medium.

Since *B. cereus* is commonly found in a soil environment where Pi is a growth limiting factor, it is thought that SMase may act as part of a Pi retrieval-scavenging system (Guddal *et al.*, 1989).

1.5.5.4 Cereolysin AB

PC and SMase genes are arranged in tandem on the *B. cereus* chromosome and are capable of functioning together co-operatively as a cytolytic unit known as cereolysin AB. Currently no specific role for cereolysin AB in *B. cereus* virulence has been established; however, it is possible that the components of cereolysin AB damage or lyse cells through the production of inflammatory mediates, that then contribute to tissue damage (Beecher and Wong, 2000b; Gilmore *et al.*, 1989).

1.5.6 Regulation of *B. cereus* toxin genes

Studies have identified PlcR as a pleiotropic regulator of the *B. cereus* group extracellular virulence factors. PlcR was first identified in *B. thuringiensis* as a gene that positively regulated the transcription of *plcA*, (encoding PI-PLC), at the onset of stationary phase growth. The PlcR protein was calculated to have a molecular weight of 33 762 and was shown to be distinctly related to PreL and NprA regulator proteins of *Lactobacillus sp.* and *B. stearothermophilus*. The *plcR* gene translation was also shown to be self-regulating (Lereclus *et al.*, 1996).

In addition to *B. thuringiensis*, PlcR has also been detected in *B. cereus* and further studies have reported that PlcR regulates several other extracellular virulence factors. These virulence factors include the degradative enzymes, cell surface proteins and a number of enterotoxins, including HBL and NHE (Agaisse *et al.*, 1999). In addition, disruption of PlcR in *B. cereus* group strains has been shown to reduce haemolytic, lecithinase and cytotoxic properties of these organisms (Salamitou *et al.*, 2000; Slamti *et al.*, 2004). Studies have also shown that the *plcR*-regulated genes are widely dispersed on the chromosome and PlcR regulation is believed to be restricted to members of the *B. cereus* group (Agaisse *et al.*, 1999). Furthermore, studies have also shown that the *plcR* gene is present in *B. anthracis,* however is inactive due to a

nonsense mutation. It is believed that this knock out may be responsible for a number of phenotypic differences between *B. anthracis* and the other members of the *B. cereus* group, including the fact that it is non-motile and non-haemolytic, as well as producing significantly lower amounts of degrading enzymes (Mignot *et al.*, 2001).

The exact mechanism by which PlcR regulation acts is still unknown, however it has been shown that oligopeptide permease (Opp) is required for the expression of the *plcR* regulon. Opp is required for the import of small peptides into the cell and it is believed that *plcR* expression is activated by the uptake of a signal peptide via Opp (Gominet *et al.*, 2001). This signalling peptide has since been identified as PapR, a 48 amino acid peptide that is secreted then re-imported via Opp. Once inside the cell, the processed peptide activates the *plcR* regulon allowing the binding of PlcR to its target DNA. Furthermore, the PapR peptide has been shown to be encoded by the *papR* gene, that is located downstream of *plcR*. Disruption of the *papR* gene has been shown to abolish expression of the *plcR* regulon (Slamti and Lereclus, 2002).

1.6 Stress response of *Bacillus* species

1.6.1 Environmental stresses on *Bacillus*

As mentioned previously (see section 1.2) *B. cereus* and *B. anthracis*, like many other bacilli, are commonly found in the soil. This natural environment has been shown to lead to a variety of stresses including; nutrient limitation, desiccation and osmotic shock, UV stress and extremes of heat and pH. In addition, *B. cereus* has also be reported to be found in groundwater and on plants and animals at the point of harvest or slaughter (Beuchat *et al.,* 1997), hence *B. cereus* is also exposed to further natural stresses in these environments. A further natural niche for *B. cereus* has been reported to be the gut microflora of invertebrates, (Jensen *et al.*, 2003). This environment has the potential of being nutrient limited, as well as containing numerous other bacteria, therefore creating additional stresses for *B. cereus.*

Studies with *B. anthracis* have shown that it is more dependent on sporulation as a survival mechanism than other Bacilli. *B. anthracis* undergoes sporulation in order to survive the adverse environmental conditions found in the soil. Since *B. anthracis* has very specific nutritional requirements, it is unlikely to germinate in the soil and therefore its vegetative multiplication phase occurs in the host, making *B. anthracis* an obligate pathogen (Turnbull, 2002). When *B. anthracis* vegetative cells are released from a dead or dying animal to the environment (normally the soil), the rate and success of sporulation of these cells is dependant on the environment, with temperature, pH, oxygen availability, sunlight, organic matter and the presence of certain cations all influencing the process.

In addition to its survival in the natural environment, *B. cereus* has also been shown to be a common contaminant in the food industry (Drobniewski, 1993). It is believed that the ability of *B. cereus* to cope with processing stresses (including heat treatment during sterilisation and pasteurisation, freezing, ethanol treatment, salt treatment, production or addition of acid and treatment of packing materials with hydrogen peroxide), may contribute to the survival of the cells during food processing and storage (Browne and Dowds, 2001, Browne and Dowds, 2002, Ivanova *et al.*, 2003).

Furthermore, *B. cereus* has also been shown to be a major contaminant of many raw and processed foods (including rice, milk and dairy products) (de Vries *et al.*, 2005). Since a number of these foods are stored under refrigeration, *B. cereus* also has to be able to withstand the stresses of low temperatures.

It has been shown that bacteria also encounter many stressful conditions in vivo; these include acid shock in the stomach, as well as phagocytosis by the immune system (Browne and Dowds, 2002). Since *B. cereus* is a known food pathogen, it has to cope with the stresses that it encounters in the gastrointestinal tract upon consumption in order to survive (van Schaik *et al.*, 2004a). Furthermore it is possible that this generation of a stress response is a prerequisite for virulence since this relationship has been reported for other pathogenic bacteria (Gahan and Hill, 1999). Studies with *B. anthracis* have also hypothesised that the ability of the vegetative cell to form a stress response after phagocytosis also influences *B. anthracis* virulence and ability to cause infection (Guidi-Rontani *et al.*, 1999a; Jensen *et al.*, 2003).

1.6.2 General stress response

Due to the above mentioned stresses encountered by both *B. anthracis* and *B. cereus*, these organisms, along with numerous other bacteria, are known to have developed a number of general stress responses (GSR). These responses allow the bacteria to continue to survive and maintain cell viability when they encounter hostile environmental conditions (such as heat, pH, osmotic and alcohol shock as well as specific nutrient starvation) (Abee and Wouters, 1999; Siegele and Kolter, 1992; Völker *et al.*, 1999) and then resume rapid growth when conditions become favourable. In addition, it has been reported that the up-regulation of starvation and stress specific proteins may allow cells to neutralise the stress factor, adapt to its presence, or repair the damaged caused by the stress (Hecker and Völker, 1998).

Different bacteria have been found to respond to stress in differing ways. Some species, including bacilli, are capable of forming dormant spores, where as others form fruiting bodies and multicellular aggregates (Siegele and Kolter, 1992). Since

bacilli have also been reported to form biofilms and fruiting bodies under some stress conditions (Branda *et al.*, 2001; Hamon and Lazazzera, 2001; Oosthuizen *et al.*, 2001) (see section 1.7), it is likely that the bacterial stress response and the phenotypic changes seen are dependent both on the organism and the nature of the stress imposed.

Numerous studies have been completed investigating the GSR of the *Bacillus* family and in particular *B. subtilis* (Hecker and Völker, 1998). Studies have shown that when growth arrest occurs through starvation (by glucose, phosphate or oxygen), or by heat, salt, ethanol or acid stress, one of the initial responses is the increased production of general stress proteins (GSPs) (Hecker and Völker, 1998). In addition, each stress has been shown to lead to the production of a unique set of GSPs (Hecker and Völker, 1990; Hecker *et al.*, 1996; Hecker and Völker, 1998). These proteins have been shown to be regulated by σ^B, an alternative sigma factor that binds RNA polymerase to direct the transcription of over 100 genes (Petersohn *et al.*, 2001). The regulatory expression of σ^B in *B. subtilis* has been extensively studied. It has been shown that there are two pathways that lead to the activation of σ^B. The first of these is induced by environmental stress conditions such as osmotic shock and ethanol exposure whilst the second pathway is due to nutrient limitation. Further research into the nutritional stress response of *B. subtilis* has revealed that activation of σ^B occurs via RsbP phosphatase. The mechanism by which RsbP is activated is currently unknown, however its activation has been shown to coincide with culture conditions that are believed to reduce the levels of high energy nucleotides in the cell, such as glucose or phosphate limitation, suggesting that changes in ATP levels may act as the activation signal for RsbP (Zhang and Haldenwang, 2003).

Studies into the stress response of *B. cereus* and *B. anthracis* have also identified σ^B in both organisms. The alternative sigma factor has been shown to be up-regulated in *B. anthracis* during heat shock and stationary phase growth. It has also been implicated in virulence, since a *sigB* null mutant has been shown to be attenuated in a mouse model (Fouet *et al.*, 2000).

The alternative sigma factor σ^B in *B. cereus* has been shown to be located in a cluster along with 3 predicted σ^B regulators and an unknown protein (van Schaik *et al.*, 2004a). In addition, a number of σ^B dependant genes have been identified in *B. cereus*, the function of which suggest that σ^B not only regulates genes directly involved in the stress response, but may also have a role in regulating metabolic rearrangements (van Schaik *et al.*, 2004b). It should however also be noted, that unlike *B. anthracis*, a *B. cereus* σ^B null mutant has been shown to produce comparable levels of virulence factors and enterotoxins as the parental wild type strain, inferring that *plcR* regulation is independent of σ^B (van Schaik *et al.*, 2004a).

As mentioned earlier, *B. cereus* is a major contaminant in the food industry and a number of mild treatments are used to reduce contamination during food processing. Studies have shown that the use of these mild treatments leads to cross-protection of *B. cereus* and may actually increase the survival of *B. cereus* in foods (Periago *et al.*, 2002). These studies have shown that when *B. cereus* cells are exposed to mild treatments, such as the exposure to temperatures of 42°C, 4% ethanol, acid stress, 2.5% NaCl or hydrogen peroxide (Browne and Dowds, 2001; Browne and Dowds, 2002; Periago *et al.*, 2002; van Schaik *et al.*, 2004a), an adaptation process is initiated that increases resistance to lethal stresses such as an acid stress of pH 4.6 or temperatures of 50°C. It has been shown that this adaptation requires the synthesis of de novo proteins that include; stress proteins, sporulation proteins and metabolic enzymes. It has therefore been hypothesised that adaptation by *B. cereus* involves a range of cellular processes (Periago *et al.*, 2002). Furthermore, it has also been shown that heat resistance and stress adaptation of *B. cereus* is regulated by the alternative sigma factor σ^B (van Schaik *et al.*, 2004a). In addition, further studies on *B. cereus* σ^B activation have revealed a novel regulator, RsbY. This regulator is located downstream of the *sigB* operon and has been shown to be up-regulated during heat shock, osmotic up-shock and ethanol exposure. In contrast to this, studies investigating the role of σ^B under ATP depletion have shown only a limiting activation of σ^B (less than 2.5 fold) when cells are depleted of ATP, strongly inferring

that σ^B activation is not triggered by energy depletion and hence nutrient limitation in *B. cereus* (van Schaik *et al.*, 2004a). These data therefore infer that *B. cereus* and *B. subtilis* have differing regulation mechanisms in response to nutrient stress and therefore, the results seen in the numerous studies with *B. subtilis* may not be applicable for *B. cereus.*

1.6.3 Sporulation

In addition to the production of a GSR, bacilli are also able to under go sporulation in unfavourable environments. The sporulation processes of Bacilli have been extensively studied and *B. subtilis* sporulation has been shown to be regulated by both Spo0A and a number of alternative sigma factors (Piggot and Hilbert, 2004). The alternative sigma factor σ^A, has been shown to be the primary sigma factor, however this is not involved in sporulation but is responsible for regulating macromolecular synthesis as well as playing a key role in the housekeeping functions of the cell (de Vries *et al.*, 2004). The alternative sigma factor σ^H, along with Spo0A, has been shown to be active in pre-divisional cells, leading to the expression of factors important for axial filament formation, asymmetric division and compartmentalisation of gene expression. After the asymmetric division, *B. subtilis* sporulation is believed to be orchestrated through the expression of four alternative sigma factors; σ^E, σ^F, σ^G and σ^K. The activation of σ^E and σ^F has been shown to occur after the division of the prespore and mother cell, whilst σ^G and σ^K are active after the engulfment of the mother cell (Hilbert and Piggot, 2004; Hilbert *et al.*, 2004).

No literature is currently available regarding Spo0A in *B. cereus*, however reports exist referring to its presence in both *B. anthracis* and *B. thuringiensis* (Lereclus *et al.*, 2000; Worsham and Sowers, 1999) and genome analysis using the EMBL-EBI databases (November 2004) reveals its presence in the *B. cereus* ATCC 14579 genome. Furthermore, comparative analysis has also predicted the *B. cereus* genome to contain single homologs to sigA, sigB, sigF and sigG responsible for housekeeping, stress response and sporulation. The expression of these sigma factors (σ^A, σ^B, σ^F and σ^G) has been studied and their kinetics have been shown to follow the model of *B.*

subtilis sigma factor expression, implying a conservation of the sporulating mechanisms amongst the *Bacillus* family (de Vries *et al.*, 2004).

1.6.4 Influence of nutrient limitation on cell physiology

It has been proposed that the unfavourable conditions imposed by stress and starvation are the rule in natural conditions. Therefore nutrient limited cultures impose a stress on bacteria that more accurately reflects the natural environment of cells, compared with complex laboratory media (Kolter, 1999). Studies into the nutrient requirements of both Gram-positive and Gram-negative bacteria have shown that starvation of cells can influence the physiology of the cell in a number of ways, depending on the nature of the starvation, as well as the strain studied.

In terms of Gram-negative bacteria, studies with *Escherichia coli* have shown that starvation leads to changes in cell morphology, with the cells becoming much smaller and spherical (Lange and Hengge-Aronis, 1991; Siegele and Kolter, 1992). The properties of the cell surface have also been reported to change when cells transition between growth and stationary phase due to starvation, with the surface of some cells becoming more hydrophobic, with an increased ability to adhere to surfaces. Furthermore, studies have also demonstrated that the sensitivity of *E. coli* to chlorhexidine can also vary under different nutrient limitations (Wright and Gilbert, 1987). In addition, nutrient limitation studies in *Burkholderia* (previously *Pseudomonas) cepacia* have shown that cells grown under varying nutrient limitations have differing levels of sensitivity to engulfment and killing by human polymorphonuclear leuckocytes (Anwar *et al.*, 1983) and studies with *P. fluorescens* have shown that nutrient limitation can also influence the attachment of cells to surfaces (Lindsay and von Holy, 1998).

A number of studies have also been completed using Gram-positive bacteria and it has been shown that the Gram-positive envelope varies with growth rate and nutrient limitation (Brown and Williams, 1985). In addition. studies with *Listeria*

monocytogenes have also shown that under nutrient limitation, viable cells become smaller.

More specifically, a number of studies into the effect of nutrient limitation have also been completed using bacilli and these studies have shown that nutrient limitation can influence both vegetative cells and spores. Studies with *B. cereus* have cited that phosphate limitation is the major growth-limiting factor in natural habitats (Guddal *et al.,* 1989). Since studies with *B. megaterium* have shown that phosphate limitation leads to a decrease in intracellular levels of protein, RNA, poly-3-hydroxybutyrate, carbohydrate and oxygen uptake in comparison with phosphate-plentiful cultures, it is therefore likely that the natural environment leads to these phenotypic changes in most bacilli. Furthermore, additional studies have also shown differences in cell structure under phosphate limitation, with phosphate-limited cells showing an absence of a thick rigid cell wall (de la Rubia *et al.*, 1986). Studies with *B. licheniformis* have shown that under phosphate limitation the cell wall composition changes form tecichoic acid (the phosphorous containing anionic polymer) to teichuronic acid (a non phosphorous containing polymer) (Kruyssen *et al.*, 1980), hence it can be seen that there are major changes in cell physiology under phosphate limitation.

Since bacteria are carbon based organisms, it can also be seen that carbon limitation would influence members of the *Bacillus* genus. Studies into the effect of carbon starvation on Bacilli have shown a wide variation in the carbon requirements of the *Bacillus* genus. Studies with *B. cereus* have shown that the organism does not have complex nutritional requirements, is able to grow in soil with low nutrient amounts (Kotiranta *et al.,* 2000) and can use either glucose, glutamate, lactate or amino acids as a carbon source (de Vries *et al.,* 2005); however, in contrast to these observations, *B. stearthermophilus* has been reported to have complex nutritional requirements with the need for many vitamins and amino acids in order to grow (Lee *et al.,* 1982).

Studies using *B. megaterium* have shown that the nature of the carbon source can affect the size and chemical composition of both spores and vegetative cells (Hitchins

et al., 1972). In addition studies with *B. subtilis* have revealed the cessation of synthesis of almost 400 proteins under glucose starvation, along with the synthesis of 150 de novo proteins including proteins involved in the general stress starvation response and proteins involved in the glucose specific starvation response. This latter group of proteins was found to lead to a drop in glycolysis along with utilisation of alternative carbon sources (Bernhardt *et al.*, 2003). Taken together, these data therefore demonstrate changes in cell physiology under varying carbon sources and carbon limitation.

Further compounds shown to have an effect on *Bacillus* cell physiology when found in limiting concentrations are iron, magnesium and manganese. Iron has previously been shown to be an essential nutrient for almost all microorganisms (Guerinot, 1994), however the availability of iron in nature is limited due to its rapid oxidation under aerobic conditions. Therefore in order to obtain sufficient quantities of iron, bacteria secrete low molecular weight iron chelators (siderophores) into the environment (Baichoo *et al.*, 2002). Further to these finding, studies with *B. anthracis* have shown that siderophore biosynthesis is required for growth in macrophages where iron is believed to be a limiting component (Cendrowski *et al* 2004). Studies into the effect of magnesium limitation on bacilli have shown that *B. subtilis* requires magnesium ions for ribonuclease P RNA activity. (Beebe *et al.*, 1996). In addition it has also been stated that some enzymes specifically require magnesium ions as a cofactor. Studies into the effect of manganese limitation on bacilli have shown that manganese is an essential nutrient required for the sporulation of bacilli and can replace magnesium as a cofactor for many (but not all) enzymes (Oh and Freese 1976).

Studies into the effect of nutrient limitation on sporulation of bacilli have shown that the nature of the nutrient starvation can lead to changes in the ability of *Bacillus* to sporulate. An investigation into the effects of nutrient limitation of *B. megaterium* has shown that sporulation does not occur in cells that are potassium, manganese or magnesium-limited, however it does occur under magnesium-limiting conditions

when cells are also depleted of glucose (Brown and Hodges, 1974). Similar findings have also been reported for *B. subtilis*, where spore formation occurs when cells are starved of glucose or ammonium, however is decreased when cells are phosphate or magnesium-limited (Dawes and Mandelstam, 1970).

Furthermore, studies with a range of Bacilli have shown that when sporulation does occur due to a variety of stresses, different nutrient limitations lead to variations in the spores formed, through their germination characteristics, heat resistance and spore volume (Brown and Hodges, 1974; Cheung *et al.*, 1982a; Evans *et al.*, 1997; Gonzalez *et al.*, 1999; Melly *et al.*, 2002). In addition, studies with *B. cereus and B. subtilis* have also shown that the sporulation pH and temperature also influences the degree of sporulation and properties of the spores produced, in terms of heat resistance, core water content and resistance to chemical agents (Gonzalez *et al.*, 1999; Mazas *et al.*, 1997; Melly *et al.*, 2002).

1.6.5 Influence of stress and starvation on virulence factor production

Studies with *P. aeruginosa* have shown that nutrient limitation can influence the stability and production of virulence factors of Gram-negative bacteria (Ombaka *et al.*, 1983). These results have also been shown in studies using *B. cepacia* where growth rate, oxygen availability and nutrient limitation have been found to influence virulence factor production. Data show that siderophore and protease production by *B. cepacia* are increased with elevated growth rates and oxygen availability, however are decreased under oxygen depletion. In contrast to these results, lipase production demonstrated the opposite trend (McKenney and Allison, 1995). In addition, more recent studies have also shown that *L. monocytogenes* haemolytic, lecithinase and phosphatidylinositol-specific phospholipase C virulence factor expression can also be influenced by varying environmental conditions and nutrient availability (Herbert and Foster, 2001; Lemes-Marques and Yano, 2004; Watson *et al.*, 1998).

Studies with gram-positive Bacilli have shown that nutrient limitation also has a vast effect on *Bacillus* vegetative cell responses and virulence factor production. A study into *B. thuringiensis* has shown that the nature of the limiting substrate influences

culture growth and crystal formation (Sakharova *et al.*, 1984). It has also been shown that *B. thuringiensis* produces large amounts of degrading enzymes at the end of the exponential growth phase in response to unfavourable conditions such as nutrient starvation (Lereclus *et al.*, 1996). Studies with *B. licheniformis* have also shown that the nature of the nutrient limiting component influences the amount of extracellular serine proteases and bacitracin produced, with ammonium limitation causing higher levels of serine protease production than glucose limitation (Hanlon *et al.*, 1982).

In addition, studies using *B. anthracis* have shown a variation in amino acid requirements, depending on the strain tested. Virulent strains have been shown to consume lysine, histidine, arginine and tryptophan, whereas non virulent, vaccine strains, do not consume these amino acids. It is therefore feasible that these amino acids are required for virulence in *B. anthracis* (Naimanov *et al.,* 1986).

Studies investigating the influence of nutrient limitation and environmental stress on *B. cereus* physiology have shown that production of the emetic toxin varies depending on the nutrient stress and the growth medium as well as dissolved oxygen tension (Agata *et al.*, 1999; Finlay *et al.*, 2002; Häggblom *et al.*, 2002). Furthermore, it has also be shown that emetic toxin production is increased under leucine, isoleucine and glutamic acid starvation, hence the presence of low concentrations of free amino acids (as found in rice, noodles pasta etc.) may promote the production of emetic toxin (Agata *et al.,* 1999). It has also been shown that temperature also influences *B. cereus* emetic toxin production, with maximal levels occurring at lower temperatures and no toxin production occurring at temperatures above 37°C (Finlay *et al.*, 2000), these studies also hypothesised that this lack of emetic toxin production above 37°C was due to a temperature dependant regulation of the toxin, however whether this regulation occurs at the transcriptional, translational or posttranslational level has not yet been determined.

Studies with the enterotoxins have shown that glucose, starch, pH and the presence and concentration of certain carbohydrates can also influence production in *B. cereus* (Garcia-Arribas and Kramer, 1990; Spira and Silverman, 1979). Furthermore, it has

also been shown that dissolved oxygen tension influences *B. cereus* enterotoxin production since aerated cultures are significantly more cytotoxic at 8°C than equivalent static cultures (Christiansson *et al.*, 1989). Studies onto the effect of temperature have shown that *B. cereus* is able to produce enterotoxin at temperatures between 4°C and 40°C. However it has been reported that higher levels occur at temperatures between 30°C and 40°C (Rowan and Anderson 1998; Mahakarnchanakul and Beuchat 1999), with the optimal temperature being 32°C (Fermanian *et al.*, 1996). Hence enterotoxin production does appear to be regulated by temperature but not in the same way as the emetic toxin.

Overall, these studies collectively demonstrate that bacteria produce varying levels of virulence factors under different stress and nutrient limiting conditions (Agata *et al.*, 1999; Chaussee *et al.*, 1997; Lemes-Marques and Yano, 2004; McKenney and Allison, 1995; Ombaka *et al.*, 1983). Therefore, the study of *B. cereus* and *B. anthracis* under a variety of stresses is likely to lead to an improved understanding of toxin production by both bacteria and hence may lead to an improved understanding of the vaccine production process for the anthrax vaccine.

1.7 Biofilm formation

Many bacteria are able to exhibit two modes of growth; they can be suspended planktonic cells or can exist as a sessile biofilm. It is this latter mode that is believed to reflect more accurately the natural life style for most organisms (Costerton *et al.*, 1995). Biofilms can be defined as 'structured communities of microbial cells living adherent to a surface, interface or each other' and are encased in a self-produced polymeric matrix (Costerton *et al.*, 1995; Davey and O'Toole, 2000). Studies have also shown that biofilms can comprise of both single and multiple bacterial species, with the mixed species biofilms being predominant in nature. Single species biofilms have however, been implicated in a number of infections and have been found on the surface of medical implants, demonstrating that they also have a clinical importance (O'Toole *et al.*, 2000).

It is widely recognised that biofilm formation is important in a number of bacterial diseases and numerous studies have been completed investigating why bacteria form biofilms. It has been reported that biofilm formation allows cells to form effective stress responses, remain in a favourable niche and act as a multicellular organism, allowing gene transfer and the division of the metabolic burden (Jefferson, 2004). In addition, it has also been hypothesised that biofilm formation is the default mode for bacteria and cells only exist planktonically in order to translocate.

1.7.1 Adherence

Despite the initial cause, bacteria can only form biofilms if they are capable of adhering to a surface, interface or other cells. Biofilm formation has been shown to be a complex process with numerous factors influencing the attachment of cells to surfaces. These include; the surface conditioning, growth medium, growth temperature, growth pH, electrostatic and physical interactions and bacteria cell-cell signalling (Lindsay *et al.*, 2002).

1.7.2 Swarming

In addition to adherence, swarming has also been shown to play a major role in the formation of biofilms (Sharma and Anand, 2002). Swarming exists for both Gram-positive and Gram-negative bacteria and is a form of migration that facilitates the rapid colonisation of a surface by bacteria. It has also been linked to increased antibiotic resistance and virulence factor production and studies have revealed a link between swarming associated virulence factors (including exo-enzymes and more specifically extracellular proteases) with pathogenesis (Givskov *et al.*, 1997; Walker *et al.*, 1999). A number of factors have been shown to be important in eliciting a swarming phenotype; these include cell density, nutrient content and the viscosity of the medium (Fraser and Hughes, 1999; Harshey, 1994)

1.7.3 Quorum sensing

A further factor involved in biofilm formation is cell-cell signalling or quorum sensing. Studies with *B. subtilis* have shown that quorum sensing can activate the Spo0A phosphorelay system. As described previously (see section 1.6.3), this system is involved in sporulation; however it has also been shown to be important in biofilm formation. In addition to biofilm formation, quorum sensing has also been linked to bioluminescence, antibiotic production, competence, sporulation and virulence factor production (Kaiser and Losick, 1993; Salmond *et al.*, 1995); it is therefore feasible that there is a molecular link between these traits, implying that biofilm formation may also influence toxin production.

1.7.4 EPS production

In addition, an exopolysaccharide (EPS) matrix is also required to 'cement' cells together in biofilms. Mutants unable to form EPS are unable to form biofilms, although they are still capable of attaching and forming micro colonies to a limited extent (Allison and Sutherland, 1987; Watnick and Kolter, 1999). EPS is secreted by cells already established in a biofilm and can vary greatly in composition and hence physical and chemical properties (Sutherland, 2001). EPS production has been

described for a wide range of bacteria in both planktonic and biofilm growth, including *Bacillus* biofilms (Larpin *et al.*, 2002; Osadchaia *et al.*, 2000).

1.7.5 Increased virulence of biofilms

It has been reported that EPS interacts with antimicrobial agents, protecting cells from their effects by preventing access of compounds or reducing their concentration. Studies have shown that established biofilms are able to tolerate concentrations of antimicrobials 10-1000 times greater than that required to kill equivalent planktonic cells (Hogan and Kolter, 2002; Lewis, 2001). In addition, studies with *B. cereus* biofilms have shown a similar trend, with planktonic cultures being much more susceptible to the actions of hypochloride than the equivalent biofilms (Peng *et al.*, 2002). Furthermore, biofilm formation has also been implicated as a feasible strategy to prevent desiccation. The major component of the biofilm matrix (up to 97%) is water (Zhang *et al.*, 1998), therefore EPS has been proposed to slow down water loss during desiccation (Hamon and Lazazzera, 2001). This may also be the case for Bacilli, since studies have shown that sporulation is not induced by desiccation.

Biofilms have also been shown to be extremely resistant to phagocytosis making them hard to eradicate from hosts and are better adapted to survive periods of environmental stress (Costerton *et al.*, 1999; Jefferson, 2004; Lewis, 2001). Studies have shown that EPS levels vary depending on the availability of carbon and other nutrient limiting components, implicating that increased production of EPS is part of a stress response. This also corresponds with reports showing that the slow bacterial growth associated with biofilms also promotes the production of EPS (Sutherland, 2001). In addition, EPS has also been implicated in protection of cells from environmental pressures such as osmotic shock and pH shifts (Davey and O'Toole, 2000). Taken together, these data therefore suggest a role for the biofilm as a stress response in order to become more resistant to environmental factors.

1.7.6 *Bacillus* species biofilms

Bacillus biofilms have been shown to have an environmental relevance due to their ability to cause contamination in paper machines (Kolari *et al.*, 2001) and water reclamation systems (Hu *et al.*, 2003). In addition, *Bacillus* biofilms have also been shown to be clinically important due to their detection on catheters (Banerjee *et al.*, 1988).

Studies into *B. subtilis* biofilms have also shown the development of two forms of biofilms. When cultures are incubated statically *B. subtilis* cells are able to form a pellicle, the individual cells migrate to the air-liquid interface where they proliferate as long chains of non-motile cells. These chains have been shown to be highly ordered and tightly bound, presumably by an extracellular matrix. Growth of these bundled chains then gives rise to larger structures such as fruiting body formation. In addition, the cells are also able to grow on solid surfaces. This interaction with surfaces also gives rise to distinctive features, some of which are not present in the pellicles, such as concentric rings (Branda *et al.*, 2004).

1.7.6.1 Molecular mechanisms

As with other species, numerous studies have also been completed investigating the genomic regulation of *Bacillus* biofilms. Similarly to planktonic cultures, most of the initial studies into *Bacillus* biofilm genomics have used *B. subtilis* as a model, however it should be noted that as with the planktonic studies, the mechanisms empolyed by *B. subtilis* may not be the same as those used by the *B. cereus* group and therefore may not be an accurate model of biofilm regulation for *B. cereus* and *B. anthracis*.

The *B. subtilis* studies have revealed a number of key transcription factors that are required for biofilm formation. Included in these is the sporulation transcription factor, Spo0A (Branda *et al.*, 2001; Branda *et al.*, 2004; Hamon and Lazazzera, 2001). Studies have shown that mutations in *spo0A* cause a defect in biofilm formation, since the cells are only capable of adhering to surfaces as a mono-layer instead of the three-

dimensional biofilm seen with wild type cells. These data therefore suggest that the Spo0A mutants are defective in the cell-cell interactions that are required for multicellular biofilm formation (Hamon and Lazazzera, 2001).

In addition, the alternative sigma factor σ^H has also been shown to affect biofilm formation. σ^H is not required for biofilm formation since *spo0H* mutants lacking σ^H are not defective in the initiation of biofilms; they are however unable to form fruiting bodies on the surface of the biofilms in the same way as the wild isolates. This therefore infers that σ^H mutants are defective in the latter stages of biofilm formation (Branda *et al.*, 2001; Hamon *et al.*, 2004).

1.7.6.2 *B. cereus* biofilms

Initial investigations into *B. cereus* biofilm formation involved the generation of *B. cereus* 5 strain biofilms on glass wool. Proteomic comparisons then showed some differences between biofilm and planktonic cultures as well as 2 hour old and 18 hour old biofilms, demonstrating that different growth conditions influence the proteomic profiles of *B. cereus* cultures (Oosthuizen *et al.*, 2001, Oosthuizen *et al.*, 2002).

Further studies have also revealed that YhbH is expressed after as little as two hours of biofilm culture (Oosthuizen *et al.*, 2002). YhbH displays significant sequence identity to YvyD from *B. subtilis* and is a member of the sigma 54 modulation protein family. This family of proteins are strongly induced by environmental stress and energy depletion by σ^B and σ^H (Drzewiecki *et al.*, 1998; Eymann and Hecker, 2001; Eymann *et al.*, 2001). Therefore the up-regulation of YhbH in *B. cereus* biofilms infers a role for the alternative sigma factors in biofilm regulation (Oosthuizen *et al.*, 2002). This is unsurprising since similar relationships have also been reported for both *S. epidermidis* and *S. aureus* (Knobloch *et al.*, 2001; Rachid *et al.*, 2000).

Although *B. subtilis* has been used as the model for *Bacillus* biofilm studies, it is still unclear if the genomic regulation of *B. subtilis* biofilms is the same for *B. cereus*. Genome analysis using the EMBL-EBI database (as at November 2004) reveals the

presence of *spo0A*, *abrB*, *sinR* and *sinI* in *B. cereus* ATCC 14579, inferring a degree of similarity between the *B. cereus* and *B. subtilis* biofilm regulatory pathways. However, since genome analysis did not reveal the presence of *spo0H* or a number of other known *B. subtilis* biofilm regulatory genes in *B. cereus*, it is likely that the two bacteria do vary in the exact mechanisms of regulation.

1.7.7 Biofilm summary

As described above, biofilms have been shown to be an important stress response of bacilli. Futhermore, pellicle formation and assocaitation of bacilli with charcoal have also been indicated as important aspects in the current anthrax vaccine production process. Therefore, the study of both *B. cereus* and *B. anthracis* biofilms in terms of both nutrient limitation and virulence factor production should further inform the current vaccine process.

1.8 Aims of the Study

- Develop a chemically defined minimal medium for the growth of *B. cereus* and use this medium to develop a range of specific nutrient limiting media.

- Investigate the influences of the environmental stresses found in the *B. anthracis* vaccine medium by growing *B. cereus* in a range of specific nutrient limiting media and studying the influence on the gross physiology of the *B. cereus* cells in terms of changes in sporulation, heat resistance and virulence factor production (HBL enterotoxin and proteases).

- Using the derived chemically defined medium and any required modifications, grow *B. anthracis* under a range of specific nutrient limitations and investigate the influence of nutrient limitation on sporulation, PA and LF production of *B. anthracis* in order to further clarify the current UK anthrax vaccine process.

- Investigate the differences between planktonic and biofilm growth on *B. cereus* enterotoxin production.

- Transfer the procedures developed with *B. cereus* biofilm studies to *B. anthracis* and additionally investigate the influence of charcoal and pellicle formation on toxin production.

Chapter 2 – Materials and Methods

2.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1. The strains were stored at -80°C in either Luria Bertani (LB) broth containing 50% glycerol, or using a Bacterial Preservation System (Technical Service Consultants Limited, UK). When in use, the strains were streaked onto LB agar plates (*B. cereus* and *B. subtilis*) or Blood agars plates (Biomerieux, UK) (*B. anthracis*) and were incubated at 37°C overnight before being transferred to 4°C. Colonies were then used for inoculation of growth media for up to one week before the plates were discarded and fresh plates were prepared.

Table 2.1 Bacterial strains

Organism	Strain / Genotype	Source
B. cereus	ATCC 14579 (Wild type – Type strain)	Supplied by HPA, Porton Down, UK.
B. subtilis	NCTC 3610 (Wild type)	Supplied by the National Collection of Type Cultures, UK.
B. anthracis	Sterne ($pX01^+ pX02^-$)	Supplied by HPA, Porton Down, UK.

2.2 Media

All reagents were from Sigma, UK, unless otherwise stated.

2.2.1 Complex media

Miller's LB broth was prepared according to the manufacturer's instructions. Media were solidified with 1.2 % agar (Oxoid, UK) where necessary. The media were sterilised by autoclaving at 121°C for 30 min.

2.2.2 Defined media

2.2.2.1 Semi defined medium (SDM)

The composition of SDM is shown in Table 2.2. Each component was prepared as a separate concentrated stock solution that was either autoclaved at 121°C for 30 min or sterile filtered through a washed 0.2 μm cellulose acetate filter (Sartorius, Germany). Appropriate dilutions of the stock solutions were then used in order to prepare batches of SDM.

2.2.2.2 Determination of essential amino acids

The casamino acids in the SDM media were substituted for defined amino acids. The essential amino acids required for *B. cereus* growth were therefore determined as below;

A 96 well plate (Sterilin, UK) was used to determine the essential amino acids. A total of 100 μl was added to each well. The wells contained either CDM(20) with 13.9 mM glucose (positive control), or the same CDM with each amino acid missing in turn. The negative controls contained CDM(20) containing 13.9 mM glucose but with no added amino acids. All wells were then inoculated with *B. cereus*, and the 96 well plate was incubated at 37°C overnight. The plate was then viewed and growth in each well was determined. Where no growth was apparent it was determined that missing amino acid was a vital nutrient for *B. cereus* growth. Each sample was completed in triplicate.

Table 2.2 Formulation of semi defined media - SDM

Component	Concentration
3-[N-Morpholino]-2-hydroxy-propanesulfonic acid (MOPSO) (pH 6.9 with 5 M NaOH)	50 mM
Magnesium sulphate	101 mM
Calcium chloride 6-hydrate (BDH, UK)	114 μM
Potassium phosphate	3.2 mM
Ammonium sulphate	40 mM
Manganese (II) sulphate 4-hydrate (BDH, UK)	4.48 μM
Thiamine	2.96 μM
D+ Glucose	13.9 mM
Casamino acids (Fisher Scientific, UK)	6 g / l

2.2.2.3 CDM(7)

The composition of CDM(7) is as SDM with the exception that the casamino acids were substituted for the amino acids; L-aspartic acid, L-glutamic acid, L-glycine, L-histidine, L-leucine, L-threonine and L-valine. All amino acids were added at 1 mM concentrations and were dissolved in distilled water, except for L-aspartic acid and L-glutamic acid that were dissolved in 100 mM and 30 mM hydrochloric acid (Fisher, UK), respectively. Each component was also prepared as a separate concentrated stock solution that was either autoclaved at 121°C for 30 min or sterile filtered through a 0.2 μm cellulose acetate filter (Sartorius, Germany).

2.2.2.4 CDM(20)

Two different preparations of CDM(20) were used in this study, the composition of the final CDM is shown in Table 2.3 and can be seen to contain all 20 amino acids. The other preparation used in the initial studies is as Table 2.3 but with 13.9 mM glucose in comparison to the 41.7 mM glucose used thereafter. Where necessary CDM(20) was solidified using 1.2 % agar (Oxoid, UK). All amino acids used were dissolved in distilled water, except for L-aspartic acid, L-glutamic acid, L-tryptophan and L-tyrosine, that were dissolved in 100 mM, 30 mM, 120 mM and 120 mM hydrochloric acid (Fisher, UK), respectively. Components were prepared as separate concentrated stocks that were then autoclaved at 121°C for 30 min or sterile filtered through a 0.2 μm cellulose acetate filter (Sartorius, Germany). In later studies 10 μM $FeSO_4$ was also added to the medium (where stated). $FeSO_4$ batches (1 mM) were prepared in distilled water and were sterile filtered as above. Fresh batches were prepared on each occasion used due to precipitation of iron over a prolonged period of time. A 10^2 dilution was then used when preparing CDM stock.

Table 2.3 Formulation of chemically defined media - CDM(20)

Component	Concentration
3-[N-Morpholino]-2-hydroxy-propanesulfonic acid (MOPSO) (pH 6.9 with 5 M NaOH)	50 mM
Magnesium sulphate	101 mM
Calcium chloride 6-hydrate (BDH, UK)	114 μM
Potassium phosphate	3.2 mM
Ammonium sulphate	40 mM
Manganese (II) sulphate 4-hydrate (BDH, UK)	4.48 μM
Thiamine	2.96 μM
D+ Glucose	41.7 mM
L-alanine	1 mM
L-arginine (hydrochloride)	1 mM
L-aspartic acid	1 mM
L-asparagine (anhydrous)	1 mM
L-cysteine (free base)	1 mM
L-glutamic acid	1 mM
L-glutamine	1 mM
L-glycine (amino-acetic acid)	1 mM
L-histidine (hydrochloride, monohydrate, L-α-amino-β-[4-imidazolyl] propionic acid,)	1 mM
L-isoleucine	1 mM
L-leucine (L-2-amino-4-methylpentanoic acid)	1 mM
L-lysine (L-2,6, diaminohexanoic acid, monohydrochloride)	1 mM
L-methionine	1 mM
L-phenylalanine (L-2-amino-3-phenylpropanoic acid)	1 mM
L-proline	1 mM
L-serine	1 mM
L-threonine (L-α-amino- β-hydroxybutyric acid)	1 mM
L-tryptophan	1 mM
L-tyrosine	1 mM
L-valine (L-2-amino-3-methylbutanoic acid)	1 mM

2.3 *B. cereus* planktonic growth studies

2.3.1 Growth of *B. cereus*

B. cereus was grown in 250 ml conical flasks containing 20 ml medium. The flasks were covered with foil and were grown at 37°C, shaking at 200 rpm in a 'Controlled Environment Incubator Shaker' (New Brunswick) when aerated, or else incubated statically in a Heraeus incubator.

2.3.2 Preparation of culture supernatants

Where required, 1 ml samples of *B. cereus* cultures were taken for toxin and protease assays. The optical densities (ODs) of these samples were measured as section 2.3.3 before the samples were transferred to 1.5 ml closed cap vials. These were then centrifuged at 9000 rpm for 2 min in a Minispin centrifuge (Eppendorf, UK) and the supernatants were transferred to fresh tubes. These tubes were then stored at -20°C for use in further analytical assays.

2.3.3 Determination of *B. cereus* growth

The optical densities of *B. cereus* cultures were measured on a Milton Roy 601 U.V. spectrophotometer at 600 nm using plastic disposable cuvettes with a 1 cm light path (Elkay, UK). Due to deviation from the Beer-Lambert law, at ODs greater than 0.3 samples were diluted in the appropriate medium in order not to exceed this value. Colony forming units (cfu) / ml were calculated using a cell density of 1.0 being equivalent to 7.5×10^7 cfu / ml. This value was determined experimentally; An overnight culture of *B. cereus* was diluted to known ODs between 0.0 and 1.0 in CDM(20). Samples (100 µl) were then serially diluted and plated out onto LB-agar plates in triplicate and were incubated statically overnight at 37°C. Colony counts were then completed and the relationship between cfu / ml and OD was determined (see Figure 2.1).

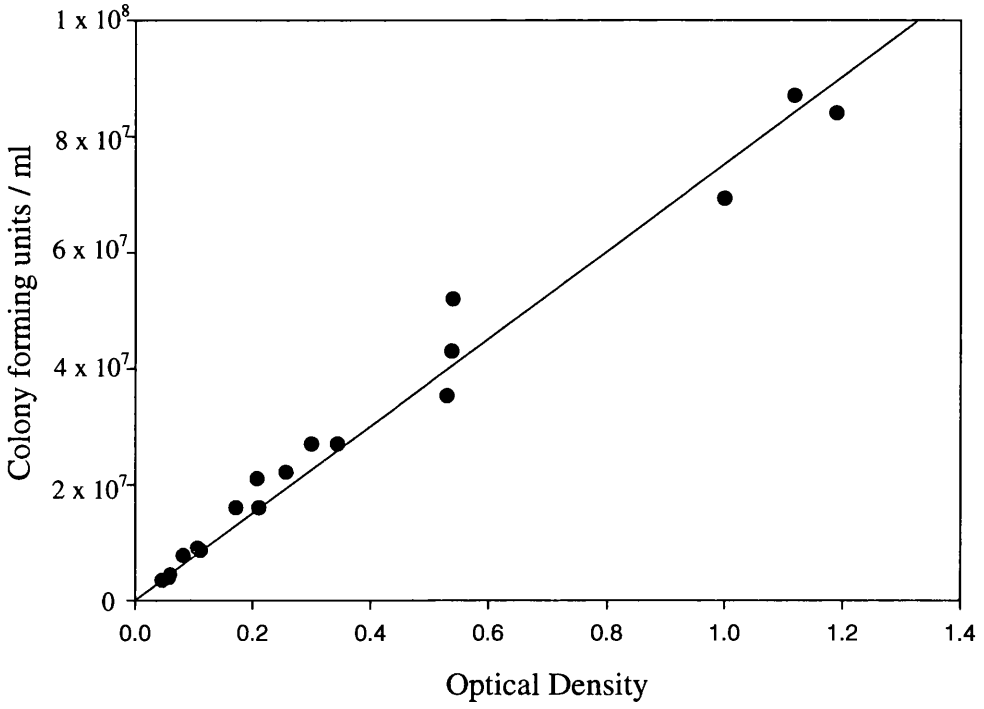


Figure 2.1 - Correlation between optical density at 600 nm of stationary phase *B. cereus* ATCC 14579 cultures and colony forming units per ml. (Combined results from three separate assays).

2.3.4 Design of growth limiting media

Nutrient limiting media were designed by growing *B. cereus* cultures overnight with varying concentrations of the limiting component. Glucose-limited cultures were determined using a range of concentrations between 0 to 55.6 mM glucose, phosphate limitation was completed using 0 to 3.2 mM K_2HPO_4, magnesium limitation was completed using 0 mM to 200 mM $MgSO_4.7H_2O$ and iron limitation was completed using 0 μM to 90 μM $FeSO_4$. The levels of manganese were also varied between 0 μM to 8.96 μM $MnSO_4.4H_2O$ in order to ascertain that the concentration used in the CDM was neither nutrient limiting nor toxic to *B. cereus* growth. Upon entry into stationary phase (16 h incubation), the optical densities of all of the cultures were measured and OD against concentration was plotted. In addition, oxygen limitation was achieved by incubating CDM cultures under static conditions and further iron limitation was achieved through the addition of diethylenetriaminepenta acetic acid (DTPA). This chelating agent was added to cultures at concentrations between 0 μM and 90 μM in order to achieve low-density iron limitation.

2.4 *B. anthracis* growth studies

2.4.1 *B. anthracis* growth conditions

All work using *B. anthracis* was completed under category 3 containment conditions at the Health Protection Agency, Porton Down. Where used, *B. anthracis* was plated out as described in section 2.1. Single colonies were then inoculated into cloning tubes containing 3 ml of the appropriate medium. These tubes were incubated at 37°C overnight, shaking at 150 rpm in an environmental shaker (Innova 4230, New Brunswick). The ODs of the cultures were measured using a Ultrospec II (Amersham Pharmacia, UK) and the cultures were diluted in the corresponding medium to give a final OD of 0.002 in 90 ml medium. These cultures were then incubated in plastic 2.5 l flasks containing a foam bung at 37°C, either shaking at 150 rpm or statically (where stated). At set time points 2 ml samples were taken and the ODs were measured. The samples were then sterile filtered through a 0.2 μm cellulose acetate filter (Sartorius, Germany) and stored at –20°C.

2.4.2 Sterility testing of *B. anthracis* supernatants

Prior to removal from the category 3 laboratory, a sterility assay was completed on the *B. anthracis* culture supernatants to ensure all cells had been removed during the filtration step. Briefly, 100 μl filtered supernatant was added to 3 ml Brain Heart Infusion in cloning tubes. These tubes were then incubated at 37°C statically for over 48 h and were then visually assessed for signs of growth. In the samples where no growth was apparent it was deemed that the samples were sterile and were removed from category 3 containment to be used in further studies.

2.5 Determination of physiological effects

2.5.1 Sporulation

2.5.1.1 Cell preparation

B. cereus was grown in a range of complete and nutrient limited media either under aeration or statically at 37°C for 24 h or 48 h. At the corresponding time points, the ODs of the cultures were measured and 9 ml culture was transferred to a separate universal tube. One ml 2.2% formaldehyde was then added to each tube in order to fix the cells and the samples were then stored at 4°C until required.

2.5.1.2 Spore counts

Aliquots of the fixed *B. cereus* cultures were diluted to an optical density of approximately 0.05 using 2.2% formaldehyde. Ten µl samples were then loaded onto an improved Neubauer 0.1 mm depth haemocytometer with a glass coverslide (Weber, UK) and were left to settle for 5 min. The slides were then viewed under 40x magnification on a Nikon Optiphot-2 light microscope (Nikon, Japan).

2.5.1.3 Spore photos

Photographs of the fixed *B. cereus* cultures were then taken using a Nikon Coolpix 995 camera (Nikon, Japan) attached to a Nikon Optiphot-2 light microscope (Nikon, Japan). All samples were placed on a glass microscope slide with a cover glass (BDH, UK) and viewed under 60x magnification using oil immersion.

2.5.2 Heat kill assays

Overnight cultures of *B. cereus* in various nutrient limited media were used in heat kill assays. The optical densities of overnight cultures were measured and the cultures were then diluted to OD 1.0 in the corresponding medium. The corresponding medium (18 ml) was then preheated in a sterile test tube at 50°C. Two ml OD 1.0 culture was then added to give a final OD of approximately 0.1. Test tubes were then incubated at 50°C for the duration of the time course. At set time points, 0.5 ml volumes were removed from the test tubes and a series of 10^1 dilutions were completed (100 µl culture into 900 µl of the corresponding medium at room

temperature). The appropriate dilutions were then plated out (100 µl) in triplicate onto LB-agar plates. These plates were then incubated at 37°C for 3-4 h before being moved to room temperature overnight. Colony counts were then completed on the plates and the appropriate graphs were plotted. A second 18 ml test tube was also prepared for each culture medium for the 0 min time point. This was treated as above except the medium was not pre-warmed at 50°C prior to dilution and plating out.

2.5.3 *B. cereus* HBL enterotoxin assays

The amount of toxin in *B. cereus* cultures was determined using a BCET-RPLA kit supplied by Oxoid, UK. Toxin levels were determined according to manufacturer's instructions. Briefly, culture supernatants were double diluted down a 96 V-bottomed well plate (Fisher Scientific, UK) using the dilution buffer supplied. Positive and negative controls were prepared in wells either containing 25 µl enterotoxin control (as supplied) or dilution buffer alone. A corresponding volume (25 µl) of either sensitised latex antibodies or latex control antibodies was then added to the wells and the samples were mixed using a pipette. Once the plate had been completed, the samples were then covered and left statically at room temperature for 22 h before being analysed. Samples showing signs of aggluation were then deemed to be positive.

Since the enterotoxin control provided with the kit contained an unknown concentration of toxin, quantification was completed based on the detection limit of the kit being 2 ng (as stated in the manufacturer's instructions). The amount of toxin present (in ng) was therefore calculated as 2x the last serial dilution at which the toxin could be detected.

2.5.4 *B. cereus* protease assays

2.5.4.1 Fluorescence assays

Initial protease assays were completed using a method developed by Twining (Twining, 1984). Briefly, reaction mixes were prepared in 1.5 ml vials containing 20 µl assay buffer (100 mM phosphate buffer pH 7.8 + 150 mM NaCl), 20 µl 5 mg /

ml casein FITC (prepared in assay buffer and stored in foil at 4°C) and 10 µl culture supernatant (either neat or after a 10^1 dilution in assay buffer). Controls were prepared using the addition of 10 µl assay buffer instead of culture sample (negative control) or 10 µl *'Streptomyce griseus* protease' instead of culture sample (positive control). The positive controls were prepared using a range of dilutions in order to generate a standard curve; proteases were added at concentrations ranging from 0 – 100 pg protease.

The samples were then incubated at 37°C in a water bath for 60 min. The reaction was then stopped by adding 120 µl 5% trichloroacetic acid and the samples were then incubated for a further 60 min at room temperature. The vials were then centrifuged at 12000g for 5 min in a Minispin centrifuge (Eppendorf, UK). Sixty µl of each supernatant was then added to 2940 µl 500 mM Tris buffer pH 8.5 and the fluorescence was measured with an excitation wavelength of 490 nm and an emission wavelength of 525 nm using a Photon Technology spectrofluorimeter (UK).

2.5.4.2 Spectrophotometer assays

Due to the variable nature of results found when using the fluorometer, later protease assays used a 'Protease Assay Kit' developed by Calbiochem. This assay is based on the initial method used by Twining (Twining, 1984), but contains a number of modifications. The method used for detecting the amount of protease activity in the culture supernatants is described below;

Samples were prepared in 1.5 ml vials, containing 100 µl samples, 50 µl FTC-casein and 50 µl incubation buffer. Positive and negative controls were also prepared using either a 1 mg / ml trypsin in PBS stock solution (as provided) or distilled water in place of the sample. The tubes were then incubated at 37°C in a water bath for 24 h. The reaction was stopped by the addition of 500 µl 5% trichloroacetic acid after which the tubes were vortexed and incubated at 37°C for 10 min. The TCA precipitate was then pelleted by centrifugation at 12000g for 5 min in a Minispin centrifuge (Eppendorf, UK). Four hundred µl supernatant was then mixed with 600 µl assay

buffer in a disposable plastic cuvette (Elkay, UK). The negative control was then used as a blank and the ODs (492 nm) of all the samples were measured using a Milton Roy 601 U.V. spectrophotometer.

In addition, a calibration curve was also completed using the Calbiochem protease assay kit. *Bacillus* species protease control was diluted in water to known concentrations between 0 and 1.2 x 10^{-2} novo protease units / gram. A protease assay was then completed as above and a calibration curve between OD and novo protease unit / gram was plotted (Figure 2.2).

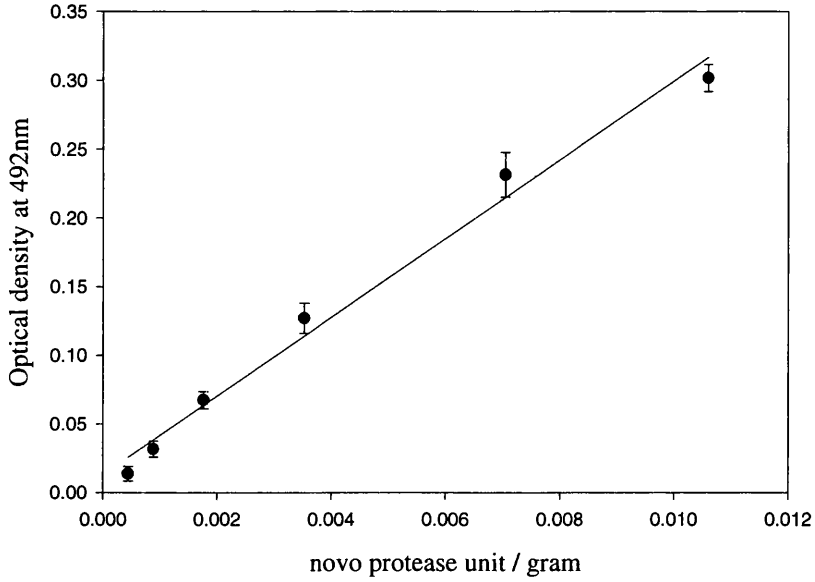


Figure 2.2 – Positive control and calibration curve between OD and novo protease units / gram for the Calbiochem protease assay kit using *Bacillus* species protease (mean ± SEM, n=3).

2.5.4.3 Protease inhibitors

A number of protease inhibitors were used in this study. These were added to the *B. cereus* cultures in order to reduce the protease activity of the samples. Initial studies used phenyl methyl sulfonyl fluoride (PMSF). This was prepared at a 100 mM concentration in ethanol (Fisher Scientific, UK) and was then sterile filtered through a 0.2 μm cellulose acetate filter (Sartorius, Germany). It was then added to cultures in concentrations up to 1 mM (as stated). The initial protease inhibitor cocktail used contained; Pepstatin A, Leupeptin, Antipain and Aprotinin. These were all reconstituted in distilled water with the exception of Pepstatin A that was resuspended in ethanol (Fisher Scientific, UK) and acetic acid (BDH, UK) at a 9:1 ratio. The proteases were added to *B. cereus* cultures at a final concentration of 10 μg / ml, with the exception of Pepstatin A that was used at a final concentration of 2 μg / ml.

Later experiments used the 'Protease Inhibitor Cocktail – P-8465' (Sigma). This cocktail contains; 23 mM 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF), 100 mM ethylenediamine tetra-acetic acid, 2 mM Bestatin, 0.3 mM Pepstatin A and 0.3 mM trans-epoxylsuccinyl-L-leucyl-amindo-(4-guanidino) butane (E64). This was prepared according to manufacturer's instructions; the solution was resuspended in 1 ml dimethyl sulfoxide and 4 ml deionised water. The inhibitor was then added to *B. cereus* cultures in volumes between 0 μl and 40 μl as stated. Protease inhibition experiments were also completed using EDTA. A 100 mM stock solution was prepared in distilled water and was added to 20 ml *B. cereus* cultures in volumes ranging from 0 μl to 20 μl.

2.5.5 *B. anthracis* toxin ELISA capture assays

PA and LF toxin assays, as developed by the HPA (Porton Down), were performed on the *B. anthracis* culture supernatants as below;

2.5.5.1 PA toxin assay

A 96 well Immulon 2 plate (Dynex, UK) was coated with 2 μg / ml antiPA (Gift from HPA, Porton Down) in phosphate buffered saline (PBS) and was incubated at 37°C

for 5 min before being transferred to 4°C for 18 h. The plate was then washed 3 x with 300 µl wash buffer (PBS-0.1%Tween) and was blocked with the addition of 100 µl blocking reagent (5% Foetal Calf Serum (FCS) in PBS-0.1%Tween). The plate was then incubated at 37°C shaking for approximately 100 min before being washed as before. One hundred µl samples, negative control or PA calibration controls (Gift from HPA, Porton Down) were added to the plate and the plate was then once again incubated at 37°C shaking for 60 min. The plate was then once again washed as before prior to the addition of 100 µl 1:2000 dilution of PA-HRP conjugate (Gift from HPA, Porton Down) to each well. After incubation at 37°C shaking for 60 min, the plate was washed once more and 100 µl pre-warmed TM Blue was then added to each well. The plate was then incubated for a further 30 min before the final addition of 50 µl 2M H_2SO_4 (BDH, UK) to each well. The plate was then read at 450 nm on a MultiSkan EX plate reader using Ascent software.

2.5.5.2 LF toxin assay

A 96 well Immulon 2 plate (Dynex, UK) was coated with 2 µg / ml antiLF (Gift from HPA, Porton Down) in PBS and was incubated at 37°C for 5 min before being transferred to 4°C for 18 h. The plate was then washed 3 x with 300 µl wash buffer (PBS-0.1%Tween) and was blocked with the addition 100 µl of blocking reagent (5% FCS in PBS-0.1%Tween). The plate was then incubated at 37°C shaking for approximately 100 min before being washed as before. One hundred µl samples, negative control or LF calibration controls (Gift from HPA, Porton Down) were added to the plate and the plate was then once again incubated at 37°C shaking for 60 min. The plate was once again washed as before prior to the addition of 100 µl 1:30000 dilution of LF-HRP conjugate (Gift from HPA, Porton Down) to each well. After incubation at 37°C shaking for 60 min, the plate was then washed once more and 100 µl pre-warmed TM Blue was added to each well. The plate was then incubated for a further 20 min before the final addition of 50 µl 2M H_2SO_4 (BDH, UK) to each well. The plate was then read at 450 nm on a MultiSkan EX plate reader using Ascent software.

2.6 *B. cereus* biofilm growth studies

2.6.1 Bühler method

Initial biofilm studies were completed using a modification of the Bühler method (Bühler *et al.*, 1998) as described in section 2.6.1.1.

2.6.1.1 *B. cereus* biofilm growth curves

Overnight cultures of *B. cereus* ATCC 14579 grown in CDM(20) were prepared as section 2.3.1. The cells were then diluted in fresh CDM to approximately 1 x 10^4 cfu / ml. Hydrophobic-edged nitro-cellulose (0.2 µm pore size, 45 mm diameter) filter membranes (Sartorius, Germany) were then washed aseptically with 5 ml CDM(20) and were placed onto plates containing CDM(20) solidified with 1.2% agar. The membranes were then inoculated with 50 µl diluted *B. cereus* cells and were incubated statically at 37°C for the duration of the time course. At set time points, plates were removed from the incubator and the membrane was transferred to a 25 ml universal tube containing 5 ml CDM(20). The tube was then vortexed for 30 sec in order to fully resuspend the membrane grown cells. A 1 ml sample was then transferred to a disposable cuvette (Elkay, UK), the OD of the sample was then taken and a growth curve was then plotted.

2.6.1.2. Sample preparation for toxin assays

2.6.1.2.1 Enterotoxin control samples

Positive controls were also prepared for analysis in toxin assay. Briefly, CDM(20) washed membranes were placed onto solidified CDM(20) plates. These were then inoculated with either 50 µl enterotoxin control or 50 µl 1 x 10^4 cfu / ml *B. cereus* in CDM(20). The plates were then incubated at 37°C for 48 h before the membranes were harvested in 5 ml M9 salts solution and were vortexed for 30 sec. The membrane and agar samples were then prepared as described in sections 2.6.1.2.2 and 2.6.1.2.3, respectively.

2.6.1.2.2.Membrane samples

Toxin assay samples were also prepared from the growth curve and control plates. Briefly, 1 ml samples were removed from the tubes containing the resuspended membranes and transferred to 1.5 ml closed cap vials. These tubes were then centrifuged at 9000 rpm / 2 min in a Minispin centrifuge (Eppendorf, UK) prior to the transfer of supernatants to fresh tubes. The samples were then stored at –20°C before toxin assays were completed as section 2.5.3.

2.6.1.2.3 Agar wash samples

In addition, toxin assay samples were also prepared from the agar plates. After the membranes had been transferred to a fresh tube for re-suspension, 5 ml M9 salts solution was added to each plate. The plates were then lightly shaken at room temperature for 90 – 120 min on a gyro-rocker (Stuart Scientific, UK) after which a 1 ml sample was removed from each plate. These samples were then stored at -20°C prior to use in a toxin assay (as section 2.5.3)

2.6.1.3 Nutrient limited *B. cereus* biofilms

Nutrient limitation was completed by growing *B. cereus* biofilms overnight but with varying concentrations of the limiting component in the solidified media. Briefly, planktonic *B. cereus* cultures were grown at 37°C for 16 h (as section 2.3.1) in CDM(20) with the omission of the nutrient-limiting component (either phosphate, magnesium or glucose). The cultures were then diluted in CDM(20) without the limiting component, to approximately 1 x 10^4 cfu / ml. Nitro-cellulose membranes (Sartorius, Germany) were prepared (section 2.6.1.1) using 5 ml of the corresponding nutrient limited CDM(20) for washing. The membranes were then placed onto plates containing solidified CDM containing varying amounts of the limiting nutrient. The glucose limited cultures were grown using a range of concentrations between 0 mM to 55.6 mM glucose, the phosphate limitation was completed using 0 mM to 6.4 mM K_2HPO_4 and magnesium limitation was completed using between 0 mM to 202.8 mM $MgSO_4.7H_2O$. The membranes were then inoculated with 50 µl of the diluted *B. cereus* cultures and were incubated statically at 37°C for 48 h. The membranes were

then transferred to universal tubes and were re-suspended in 5 ml of the appropriate nutrient limited CDM(20) by vortexing for 30 sec. One ml of each sample was then transferred to a disposable cuvette (Elkay, UK) and the OD of the sample was read. OD vs concentration of the limiting component was plotted with the linear portion of the graphs revealing concentrations at which cell density was influenced by the concentration of the nutrient being studied.

2.6.2 Floating membrane method

Due to the inability to detect toxin with the modified Bühler method described in section 2.6.1, a further modification of the method was developed with the aim of both increasing toxin recovery and reducing the amount of contaminating nutrients in the media (due to the inclusion of agar). Overnight cultures of *B. cereus* ATCC 14579 were grown in CDM(20) as section 2.3.1. The cells were then diluted in fresh CDM(20) to approximately 1 x 10^4 cfu / ml. Hydrophobic-edged nitro-cellulose (0.2 µm pore size, 45mm diameter) filter membranes (Sartorius, Germany) were then washed aseptically with 5 ml CDM(20) and were gently placed onto petri dishes (Sterilin, UK) containing 20 ml CDM(20). The membranes were then inoculated with 50 µl diluted *B. cereus* cells and were incubated at 37°C for the duration of the time course. At set time points, plates were removed from the incubator and the membrane was transferred to a 25 ml universal tube containing 5 ml CDM. The tubes were then vortexed for 30 sec prior to the transfer of 1 ml to a disposable cuvette (Elkay, UK). The OD of the sample was then taken and a growth curve was then plotted.

2.6.2.1 Sample preparation for toxin assays

2.6.2.1.1. Membrane samples

Toxin assay samples were prepared from the growth curve membranes as section 2.6.1.2.2.

2.6.2.1.2 Medium samples

In addition, toxin assay samples were also prepared from the medium remaining in the petri dishes. After the membranes had been transferred to a fresh tube for re-

suspension, a 1 ml sample was removed from each dish. These samples were then stored at -20°C prior to use in a toxin assay (as section 2.5.3).

2.6.2.1.3 Enterotoxin control samples

Positive controls were also prepared for analysis in toxin assays. Briefly membranes were washed with 5 ml distilled water and were placed onto petri dishes (Sterilin, UK) containing either 4.8 ml CDM(20) or LB. Two hundred µl enterotoxin control was then added to each membrane prior to incubation at 37°C for 2 h. A 1 ml sample was then removed from either the LB or CDM(20) medium and was centrifuged at 9000 rpm for 2 min in a Minispin centrifuge (Eppendorf, UK). The supernatant was then transferred to fresh tube that was stored at 20°C. In addition perti dishes were also set up without membranes, 200 µl enterotoxin was added directly into the media and the plates were incubated at 37°C for 2 h. Samples were then taken and treated as above. Toxin assays were then completed as section 2.5.3.

2.6.3 96 well plate method

B. cereus and *B. subtilis* biofilm growth curves were also completed using a modified O'Toole method (O'Toole *et al.*, 1999). Planktonic cultures were grown in either CDM(20) or LB at 37°C for 16 h. The cultures were diluted to an approximate OD 0.010 (7.8×10^5 cfu / ml) in the corresponding medium. Two hundred µl samples or medium controls were then inoculated into a polystyrene 96 well plate (Sterilin, UK) that was then covered and incubated at 37°C. At set time points, supernatants were harvested from triplicate wells, pooled and centrifuged at 9000 rpm for 2 min in a Minispin centrifuge (Eppendorf, UK). The supernatants were then transferred to a fresh tube and were stored at –20°C until required for further analysis. After the supernatants had been removed, 200 µl fresh medium was added to each well. Twenty-five µl 2% crystal violet solution was then added to the wells and the plates were re-incubated at 37°C for 15 – 20 min. The liquid was then removed from the wells and the wells were washed 3x with 300 µl distilled water. The wells were then allowed to dry at room temperature before the addition of 200 µl 95% ethanol (Fisher

Scientific, UK). Samples (125 µl) were then transferred from each well to a fresh plate that was then read at 595 nm using a Dyntech MR5000.

2.6.3.1 Supplemented LB

In some experiments supplemented LB was used in place of either CDM(20) or LB, this was prepared as below in Table 2.4. Each component was prepared as a separate concentrated stock that was autoclaved at 121°C for 30 min. Appropriate dilutions of the stock solutions were then used in order to prepare batches of the supplemented LB.

Table 2.4 – Preparation of supplemented LB

Component	Concentration
LB	12.5 g / 500 ml
Ammonium sulphate	0.15 M
Sodium citrate (trisodium salt, dihydrate)	34 mM
Magnesium sulphate	1 mM
D+ Glucose	0.1%
Potassium phosphate	100 mM

2.6.3.2 Submerged washing

Where stated plates were also washed through submersion, in these studies, the biofilms were set up as section 2.6.3 however after incubation with the crystal violet solution, the plates were placed into a plastic container containing 1 l distilled water. The plates were then removed and emptied by inversion and blotting onto paper towels. This was then repeated twice more using fresh distilled water for the subsequent washes.

2.6.3.3 PVC plate sterilisation

Where stated polyvinyl chloride (PVC) 96 well plates (Costar, USA) were used in place of polystyrene 96 well plates. Since these were required to study bacterial growth the plates were sterilised prior to inoculation. The plates and polystyrene lids

(Sterilin, UK) were submerged in 95% ethanol (Fisher Scientific, UK) for 15 min before being left to dry, inverted, at room temperature overnight.

2.6.3.4 Log phase inoculum

In addition to inoculation with stationary phase cultures, some experiments were also conducted using logarithmic phase cells as an inoculum. Planktonic *B. cereus* cultures were grown either in LB or CDM(20) at 37°C for 16 h. The ODs of the cultures were then measured and the cells were diluted to approximately OD 0.01 in 20 ml of the corresponding medium in a 250 ml conical flask. The cells were then grown as section 2.3.1 with OD readings being completed every 30 min. These readings were then plotted against time and when the cultures had appeared to enter log phase, they were diluted for use as an inoculum as section 2.6.3. The biofilms were then also completed as section 2.6.3.

2.7 Determination of *HblC* mRNA toxin levels in *B. cereus* cultures

2.7.1 Primers and probes

The primers and probes used in this study are listed in Table 2.5. All primers used were prepared as 10 mM stocks.

Table 2.5– Primers used in this study

Primer Name	Sequence	Reference
HblC Forward	GATACCAATGTGGCAACTGC	(Guinebretière *et al.*, 2002)
HblC Reverse	TTGAGACTGCTCGCTAGTTG	(Guinebretière *et al.*, 2002)
RT Forward	ATGAAAACTAAAATAATTACAG	(Phelps and McKillip, 2002)
RT Reverse	ATCCTTTGCTTTTTGAATTTAA	(Phelps and McKillip, 2002)

2.7.2 Mini gel electrophoresis

DNA and RNA samples were analysed by electrophoresis using a Mini-sub Cell GT electrophoresis Cell system (BioRad, UK). Gels were prepared with 0.6-0.8% w/v agarose in tris borate EDTA (TBE) containing 15 µl 500 µg / ml ethidium bromide. The samples were mixed with 6x loading solution prior to loading into individual wells. In addition, a 1 kb DNA marker (Amersham Biosciences) was also added to each gel as a standard. The gels were then run at 110 Volts for 40 min using TBE as a running buffer. Gels were then viewed and photographed on a Gene Genius (Syngene, UK).

2.7.3 Cloning of *HblC*

2.7.3.1 *B. cereus* template

B. cereus ATCC 14579 was plated out onto LB-agar and was incubated at 37°C for 16 h. A colony was then transferred to 25 µl sterile water and was incubated at 95°C for 10 min in order to generate a polymerase chain reaction (PCR) template.

2.7.3.2 PCR reactions

Reaction mixes were prepared using PuReTaq Ready-to-go PCR beads (Amersham Biosciences) containing 1 µl 10 mM forward primer (either *HblC* or RT), 1 µl 10 mM

reverse primer (either *HblC* or RT), 2.5 µl template and 20.5 µl sterile water. PCR reactions were then completed using a PE7900 (Applied Biosystems), under the following conditions; 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 5 min.

2.7.3.3 Transformation of *E. coli* by heat shock

Cloning was then completed on the PCR products. Briefly, *E. coli* 'one shot' (Invitrogen, UK) was removed from storage at –80°C and thawed on ice. Reaction mixes were then prepared with 2 µl PCR product, 1 µl salt solution (Invitrogen, UK), 2 µl sterile water (Invitrogen, UK) and 1 µl TOPO vector (Invitrogen, UK). The solutions were mixed and incubated at room temperature for 5 min before being placed on ice. Two µl samples were then added to the *E. coli* cells and were incubated on ice for 5 min. The cells were then heat shocked for 30 sec at 42°C before the addition of 250 µl LB to each tube. The samples were then incubated at 37°C for at least 60 min before being plated out on to LB-agar plates containing 200 µg / ml ampicillin (either as 90 µl neat solutions or a 10 µl solution diluted with 50 µl SOC). The plates were then incubated for 16 h at 37°C.

2.7.3.4 Colony blotting

The plates were then removed to room temperature and colonies blots were completed. Briefly, colonies were selected from each agar plate and were transferred to fresh LB-agar plates containing 200 µg / ml ampillicin using sterile plastic loops. The plates were then incubated at 37°C for 16 h. The remaining cells from each colony were then added to 25 µl sterile water in PCR tubes. These tubes were then heated at 95°C for 10 min to generate a template for a PCR reaction. Samples (2.5 µl) were then transferred to PCR tubes containing PuReTaq Ready-to-go PCR beads (Amersham Biosciences),1 µl 10 mM RT-forward primer, 1 µl 10 mM *HblC*-reverse primer and 20.5 µl sterile water. PCR reactions were then completed and analysed on agarose gels as sections 2.7.2 and 2.7.3.2.

Positive samples were then noted and cultures were prepared in 2 x tubes containing 3 ml LB with 200 µg / ml ampicillin, using the corresponding colony as an inoculum. The tubes were incubated at 37°C for 16 h.

2.7.3.5 Minipreps

Minipreps were then completed on the culture samples using the Wizard Plus SV Miniprep Kit (Promega) according to manufacturer's instructions. The final DNA products were the stored at –20°C with the exception of 10 µl aliquots that were used in enzyme digests.

2.7.3.6 Enzyme digests

Reaction mixes were prepared with 10 µl DNA (from minipreps), 2 µl EcoR1 buffer (Promega), 2 µl BSA (Promega), 1 µl EcoR1 (Promega) and 5 µl sterile water. Samples were then incubated at 37°C for 2 h before being analysed on an agarose gel as section 2.7.2.

2.7.3.7 Nanodrop

The concentration (g / l) of the DNA in the miniprep products was then determined using a NB-1000 spectrophotometer. Copy number / ml estimations were then calculated assuming a 5.2 kb plasmid size (3.9 kb TOPO vector and 1.3 kb insert) as below;

$$\text{Copies / l} = \frac{\text{(concentration of DNA g/l)}}{2.86^{6}\ \text{(molecular weight of plasmid + insert)}} \times 6.022^{23}$$

2.7.4 RNA preparation

RNA extraction was completed on both planktonic and biofilm *B. cereus* growth curve samples grown in CDM(20) and overnight cultures grown in LB.

2.7.4.1 Sample preparation

One ml *B. cereus* culture samples were added to 4 ml GTC solution (5 M guanidium thiocyanate, 0.5% sodium N Lauryl sacrosine, 25 mM tri-sodium citrate pH 7.0, 0.1 M 2-mercaptoethanol and 0.5% tween 80) in a fume hood. These samples were then left to stand at room temperature for 60 min before being transferred to –20°C for storage prior to RNA extraction.

2.7.4.2 RNA extraction

One ml aliquots were centrifuged at 3000 rpm for 15 min in a eppendorf centrifuge, S417C and the pellets were resuspended in 1 ml Trizol (Invitrogen, UK) in lysing matrix B ribolyser tubes (Q-Biogene, UK). Samples were then ribolysed for 45 sec at 6.5 ms^{-1} using a Hybrid Ribolyser (Hybrid, UK). Phenol chloroform extraction was then completed on the supernatants as below; supernatants were mixed with 240µl phenol chloroform and were centrifuged at 13000 rpm for 10 min. The supernatants were then transferred to fresh tubes containing 600 µl chloroform and once again centrifuged at 13000 rpm for 5 min. This process was then repeated once more, with the final supernatants being resuspended in 660 µl isopropanol solution (BDH, UK) and transferred to –80°C for storage.

2.7.4.3 RNA 'clean up'

The samples from the RNA extraction were then centrifuged at 13000 rpm for 10 min. The supernatants were then discarded and the pellets were resuspended in 500 µl 70% ethanol (BDH, UK). The samples were then again centrifuged at 13000 rpm for 10 min and the pellets were vacuum dried for 30 min at 30°C in a eppendorf concentrator 5301. The pellets were then resuspended in 50 µl RNAse free water (Qiagen, UK) containing 2.5 µl DNAse (New England Biolabs) and were incubated for 30 min at 37°C. The samples were then treated using RNeasy MinElute Cleanup Kit (Qiagen, UK) according to manufacturer's instructions with the final RNA samples being transferred to –80°C for storage.

2.7.4.4 PCR / Reverse Transcriptase (RT)-PCR reactions

The RNA extraction method was analysed by completing both RT-PCR and PCR reactions with the extracted RNA as template. Reaction mixes were prepared using either PuReTaq Ready-to-go PCR beads (Amersham Biosciences) or Ready-to-go RT-PCR beads (Amersham Biosciences) containing 2 µl 10 mM forward primer, 2 µl 10 mM reverse primer and 1 µl RNA template (where included). Sterile water was then added to a total volume of 50 µl. Both the PCR and RT-PCR reactions were completed as section 2.7.3.2 with the initial addition of incubations at 42°C for 30 min followed by 95°C for 5 min for the RT-PCR samples. The products from both the PCR and RT-PCR reactions were then analysed on a gel as section 2.7.2.

2.7.5 Taqman Assays

2.7.5.1 Primers and probes

Primers and probes were designed using Primer Express (Applied Biosystems) and checked for specificity using FASTA (EMBL Nucleotide Sequence Database Library). They were supplied by Applied Biosystems, prepared as 100 mM stocks in sterile water and were stored at 4°C. The Taqman probe was labelled with 5'FAM and 3'TAMRA quencher.

Table 2.6 – Taqman primers and probes

	Sequence
Forward Primer	GTCACATCCATTGTAACTGGAGGAA
Reverse Primer	GCGCACCTAATTTTCGTAATGAAG
Probe	TCTCGCAACACCAATCGTTCAAGCA

2.7.5.2 Template optimisation

The RNA controls from section 2.7.4 were thawed and diluted $10^2 - 10^4$ fold in sterile water. RT-PCR reaction mixes were then prepared as follows; Ready-to-go RT-PCR beads (Amersham Biosciences) with 45 µl sterile water, 2 µl 10 mM forward primer (see section 2.7.1), 2 µl 10 mM reverse primer (see section 2.7.1) and 1 µl RNA

template. RT-PCR reactions were then completed using a PE7900 (Applied Biosystems) under the following conditions;

42°C for 30 min then 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. The final extension was completed at 72°C for 5 min and the samples were then held at 4°C.

RT-PCR products were then analysed by electrophoresis (see section 2.7.2) and the final dilution showing a product was selected for use as a control in the Taqman assays.

2.7.5.3 Primer and probe optimisation

Forward and reverse Taqman primers were prepared at 1 µM, 6 µM and 18 µM concentrations in sterile water. RNA control extracted from overnight *B. cereus* cultures grown in LB (as section 2.7.4) was also diluted in sterile water as a template for the RT-PCR reaction.

Reaction mixes were then prepared in triplicate using a SAS-1200 robot (Corbett Robotics); 0.25 µl 25 µM probe, 1.25 µl forward primer (either 1 µM, 6 µM or 18 µM), 1.25 µl reverse primer (either 1 µM, 6 µM or 18 µM), 5 µl 10^3 diluted RNA template (or 5 µl sterile water for negative controls), 0.63 µl 40x Multiscribe and RNAse inhibitor mix (Applied Biosystems), 12.5 µl 2x Mastermix (Applied Biosystems) and 4.12 µl sterile water. The 25µl samples were then added to a 96 well optical plate (Applied Biosystems). The plate was then manually sealed with a optical adhesive cover (Applied Biosystems) and briefly centrifuged to ensure all liquid was at the bottom of the wells. The plate was then placed in a 7900HT fast real time PCR system (Applied Biosystems) and a Taqman assay was completed with the following conditions;

48°C for 30 min then 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Data collection occurred during the 45 cycle stage and the Taqman assay was completed using the Generic Taqman detector.

Average CT values were then plotted for each combination of forward and reverse primer concentration. The primer combination with the lowest average CT value and error (6 μM forward primer and 6 μM reverse primer) was then selected for use in further assays.

2.7.5.4 Copy number / CT calibration

The DNA preparations from section 2.7.3 were thawed for use in a Taqman assay for the generation of a copy number / Crossing Threshold (CT) calibration curve. Serial (10^1) dilutions were completed on the DNA in sterile water. Reaction mixes were then prepared in triplicate using a SAS-1200 robot (Corbett Robotics). They contained; 0.25 μl 25 μM probe, 1.25 μl 6 μM forward primer, 1.25 μl 6 μM reverse primer, 12.5 μl 2x Mastermix (Applied Biosystems), 4.75 μl sterile water and 5 μl DNA (at 10^0 to 10^6 dilutions) or 5 μl sterile water for negative controls. The 25μl samples were then added to a 96 well optical plate (Applied Biosystems). The plate was then manually sealed with a optical adhesive cover (Applied Biosystems) and briefly centrifuged to ensure all liquid was at the bottom of the wells. The plate was then placed in a 7900HT fast real time PCR system (Applied Biosystems) and a Taqman assay was completed with the following conditions;

50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data collection occurred during the 40 cycle stage and the Taqman assay was completed using the Generic Taqman detector.

2.7.5.5 Quantification of mRNA in culture samples

The RNA samples from the *B. cereus* growth curves (planktonic and biofilm) from section 2.7.4 were thawed for use in a Taqman assay. A 10^1 dilution in sterile water

was completed on all the growth curve samples and a 10^3 dilution in sterile water was completed on the positive control (as section 2.7.5.2).

Reaction mixes were then prepared in triplicate using a SAS-1200 robot (Corbett Robotics). They contained; 0.25 µl 25 µM probe, 1.25 µl 6 µM forward primer, 1.25 µl 6 µM reverse primer, 0.63 µl 40x Multiscribe and RNAse inhibitor mix (Applied Biosystems), 12.5 µl 2x Mastermix (Applied Biosystems), 5 µl RNA or sterile water for negative controls and 4.12 µl sterile water. The 25 µl samples were then added to a 96 well optical plate (Applied Biosystems) that was then treated as section 2.7.5.3. A Taqman assay was then completed using the conditions described in section 2.7.5.3.

Average CT values were then calculated for each of the samples and copy numbers were determined using the calibration curve generated in section 2.7.5.4.

2.8 Statistical Testing

Statistical analysis was performed on the results from the heat kill assays (Chapter 3), HBL toxin assays (Chapter 4), protease assays (Chapter 4) and O'Toole biofilm studies (Chapter 5). Since none of the results obtained in these assays were normally distributed, non-parametric tests were performed on the data to test statistical significance between samples, or samples and controls.

Unless otherwise stated the Wilcoxon Signed Rank Sum Test was used to compare paired data and the Friedman Test (indicated in the text) was used to compare more than two groups of data. Any p values less than 0.05 indicated statically significant differences between the samples. It should however be noted, that since these tests only sum the number of positive and negative differences between samples and do not take into account the magnitiude of the differences between the samples, some results that are shown to be statisitcally significant may not be clinically relevant and visa versa.

All tests were completed using SPSS Version 12 (Microsoft) and Microsoft Excel software.

Chapter 3 - *B. cereus* growth conditions and physiological studies

3.1 Introduction

Many studies have been completed growing bacteria in complex media and the physiological effects of temperature, osmolarity and pH in most species are well recognised. In addition, nutrient limitation has also been shown to have vast influence on the physiology and morphology of numerous bacteria (Anwar *et al.*, 1983; Brown and Hodges, 1974; Cheung *et al.*, 1982a; Ombaka *et al.*, 1983). Unfortunately, the use of complex media, such as LB, prevents nutrient limitation studies due to the unknown components in these complex solutions. Therefore, studies have introduced chemically defined media allowing the introduction of specific nutrient limitations, leading to a better understanding of the influence of specific nutrient depletion on cell physiology.

Several chemically defined media have been derived for a number of *Bacillus* strains including *B. cereus* and *B. anthracis* (Agata *et al.*, 1999; Nakata, 1964; Ristroph and Ivins, 1983); however, to date, these media have not specifically been used to study the effects of nutrient limitation on the cell physiology of these strains. Therefore, it is still unknown if specific nutrient depletion effects sporulation, germination, heat resistance and virulence factor production of these Bacilli.

In addition, the anthrax vaccine medium developed at the Health Protection Agency, has been shown to be a chemically defined medium, with the exception of the addition of charcoal and casamino acids to the medium (Personal communication – Richard Sharp, HPA). Further studies into this medium have also proposed that the *B. anthracis* cells grown in the vaccine preparation are under both phosphate and oxygen limitations. Since nutrient limitation has been shown to influence gross physiology and virulence factor production in other species, it is likely that this medium is also having an effect on *B. anthracis*, although the exact nature of this effect is currently unknown.

The aims of this study were therefore to develop a chemically defined medium for the growth of Bacilli and to define conditions where the cells were grown under a number of defined nutrient limitations. Since *B. cereus* is a valuable surrogate for *B. anthracis* (Beuchat *et al.*, 2004) and is also capable of causing disease (Kotiranta *et al.*, 2000), this species was chosen for this study. The sporulation properties and heat resistance of the cells were examined in order to investigate how nutrient limitation affects the gross physiology of *B. cereus*.

3.2 *B. cereus* growth medium

3.2.1 Growth profile of *B. cereus* in complex media

B. cereus ATCC 14579 was initially grown in Luria Broth (LB), a complex undefined medium containing the nutrients required to support *B. cereus* growth. Figure 3.1 shows growth curves for *B. cereus* grown in a variety of media, including LB. The LB curve illustrates the main phases of bacterial growth, with the initial lag phase lasting approximately 50 minutes, the logarithmic growth phase with a doubling time of approximately 22 minutes, and the stationary phase reaching a final optical density of 10.

3.2.2 Production of a CDM for *B. cereus* growth

A number of CDM have been reported for various strains of *B. cereus* (Agata *et al.*, 1999; de Vries *et al.*, 2004; Glatz and Goepfert, 1976; Nakata, 1964); however since none of these media were prepared for the growth of *B. cereus* ATCC 14579, a novel preparation was used in this study. This CDM was designed based on the nutrients included in the *B. anthracis* UK vaccine preparation (See appendix 1, Personal communication – Richard Sharp, HPA) and the medium used for growth of *B. anthracis* in the USA vaccine preparation (Ristroph and Ivins, 1983). Supplemental additions to the medium were based on a chemically defined medium previously optimised in the lab for growth of *Pseudomonas aeruginosa*.

As in the UK anthrax vaccine medium, casamino acids were initially used as the amino acid source supporting *B. cereus* growth; however this was problematic, since

although all the salt solutions in the medium were defined, unknown trace elements were present in the casamino acids and hence the medium was only a semi-defined medium (SDM). Figure 3.1 also illustrates a growth curve for *B. cereus* in SDM. These results show that there is a longer lag phase than with the LB growth curve, with a slightly reduced doubling time. The final optical density (OD) of the culture is however, approximately the same as LB broth at OD 10. These results are expected since although SDM is believed to contain all the nutrients required for *B. cereus* growth, the 'defined' medium will not contain the unknown trace factors that are beneficial for bacterial growth, leading to an increased doubling time.

3.2.3 Determination of essential amino acids for *B. cereus* growth

To generate a CDM for *B. cereus*, the casamino acids were substituted for a mixture of defined amino acids. Therefore, in order to replace the casamino acids with specific amino acids, it was essential to establish which amino acids were required for growth of *B. cereus*. By growing *B. cereus* in media containing combinations of 19 amino acids, missing each L-amino acid out in turn, it was apparent that L-valine, L-threonine and L-leucine were essential for growth. These results were also seen in a previous study by Agata *et al* on *B. cereus* NC7401, implying a similar nutrient requirement for the two strains. The study by Agata *et al* also showed that the inclusion of L-glycine, L-aspartic acid, L-glutamic acid and L-histidine were beneficial for the growth of *B. cereus* (Agata *et al.*, 1999). Taking these data into account, the chemically defined medium was established containing 1 mM L-valine, L-threonine, L- leucine, L-glycine, L-aspartic acid, L-glutamic acid and L-histidine and was termed CDM(7). At 1 mM, L-valine, L-threonine and L-leucine were found to have no limiting effect on *B. cereus* growth in CDM.

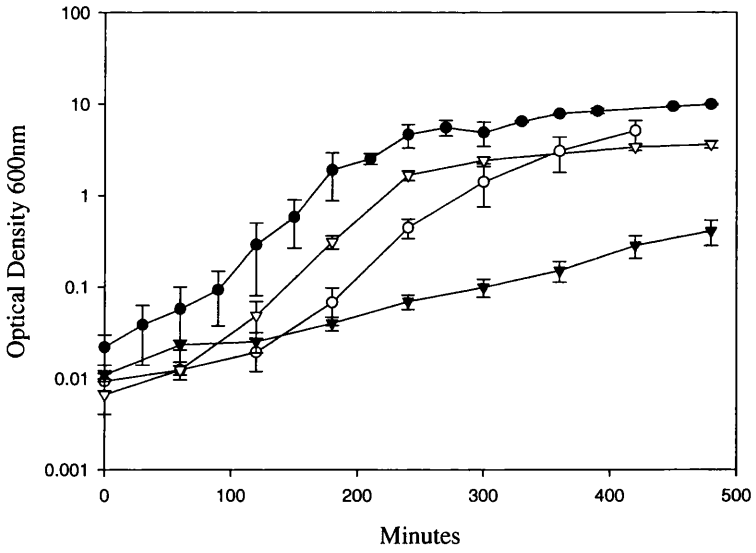


Figure 3.1 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C under aeration in LB (●), SDM (○), CDM(7) (▼) and CDM(20) (▽), (mean ± SD, n=3).

Growth curves were then completed for *B. cereus* in CDM(7) (Figure 3.1). Average data shows a lag time of approximately 200 minutes, a doubling time of 92 minutes with a final cell density of OD 2.6. From these data it is clear that the growth of *B. cereus* in this medium is vastly reduced compared to LB broth and SDM, the increased duration in the lag and doubling time also infer that the bacteria is growing under sub-optimal conditions and a beneficial nutrient is missing from the growth media.

In order to overcome this vastly reduced growth rate, the remaining 13 L-amino acids were also included in the medium, generating CDM(20). Growth curves completed with this medium showed the lag phase to be reduced (compared with CDM(7)) to approximately 35 minutes, a doubling time of approximately 26 minutes, with a final cell density of OD~4. The growth data for all conditions are summarised in Table 3.1.

Table 3.1 Summary of planktonic growth data for *B. cereus* ATCC 14579 in various media (mean ± SD, n=3).

Medium	Lag time (min)	Doubling Rate (min)	Final Cell Density OD (600nm)
LB Broth	50 ± 17.3	22 ± 1.7	9.9 ± 0.38
SDM	90 ± 30.0	28 ± 1.7	10.4 ± 1.07
CDM(7)	200 ± 17.3	92 ± 3.4	2.44 ± 0.34
CDM(20)	35 ± 8.6	26 ± 3.5	3.84 ± 1.18

3.3 Nutrient limitation of CDM(20)

The production of CDM(20) allowed the completion of nutrient limitation studies. Initial limitations were completed for phosphate, glucose and magnesium. This was achieved by varying the amount of the limiting component in the medium and recording the final overnight, stationary phase, optical density.

3.3.1. Glucose Limitation

Nutrient limitation was attempted for carbon, by varying the amount of glucose in the media. The original level of glucose in CDM(20) was 13.9 mM (as shown in Figure 3.1 and Table 3.1). Growth was studied between 0 mM and 55.6 mM glucose (see Figure 3.2). The results showed that at the 13.9 mM concentration used, the medium was bordering glucose limitation and therefore the amount of glucose added to the complete medium was increased to 41.7 mM for all subsequent studies. When no glucose was added to the medium, growth was still apparent up to OD 1.5. Growth at this level was achieved through the utilisation of the amino acids in the medium as the carbon source. Due to the high culture density without addition of glucose, low-density glucose limitation could not be achieved. The only glucose limitation therefore possible was high-density glucose limitation, for which the original concentration of 13.9 mM glucose was selected.

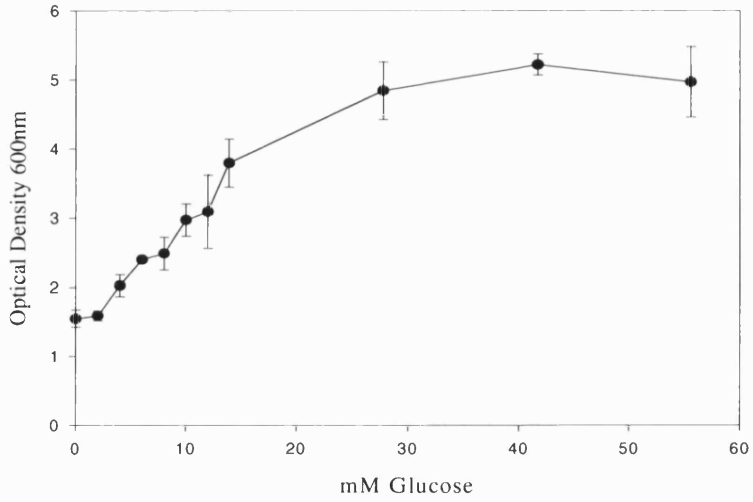


Figure 3.2 – Effect of glucose concentration in CDM(20) growth medium on planktonic stationary phase density of *B. cereus* ATCC 14579 at 37°C under aeration (mean ± SEM, n=3).

3.3.2 Phosphate Limitation

B. cereus was grown in CDM(20) ranging in concentration from 0 μM added phosphate to 3.2 mM phosphate. From the results it can be seen that phosphate limitation was achieved at concentrations up to 100 μM phosphate, after which further additions of phosphate did not increase final cell density, indicating that the cells were then limited by some other factor. Figure 3.3 illustrates the relationship between phosphate concentration and cell density. When no phosphate was added to the cultures, there was still a low level of growth; this is likely to be due to contaminating phosphate in the medium and glassware. Using these data, a 40 μM concentration was chosen for further phosphate limitation studies. This concentration is approximately midway along the linear relationship between concentration and optical density and also allows growth to a reasonable final cell density, allowing further studies on the cells and supernatants.

3.3.3 Magnesium Limitation

Cultures were magnesium-limited between 0 mM and 200 mM added magnesium, a linear correlation was seen between 0 mM and 14 mM above which tailing off was seen, followed by a plateau, implying that the cultures were limited by some other factor (Figure 3.4). When no magnesium was added to the culture, growth was seen up to OD 0.15, again likely due to contamination from chemicals and glassware. A final concentration of 6 mM magnesium was selected for further nutrient limitation studies (for the same reasons as seen above for 40 μM phosphate).

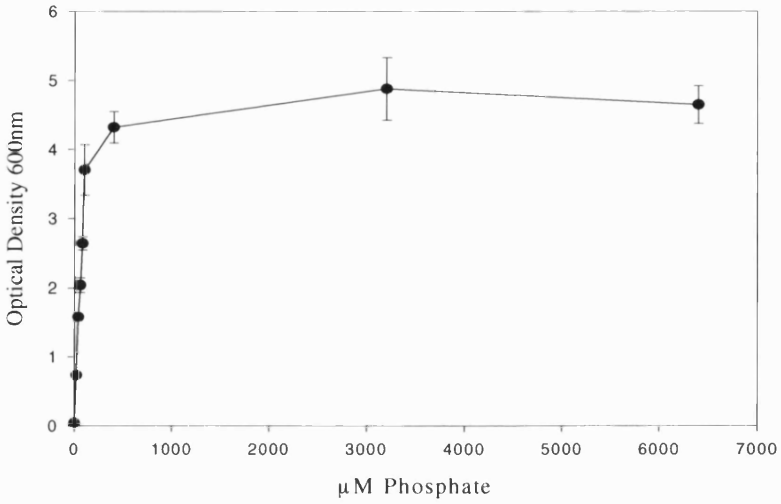


Figure 3.3 – Effect of phosphate concentration in CDM(20) growth medium on planktonic stationary phase density of *B. cereus* ATCC 14579 at 37°C under aeration (mean ± SEM, n=3).

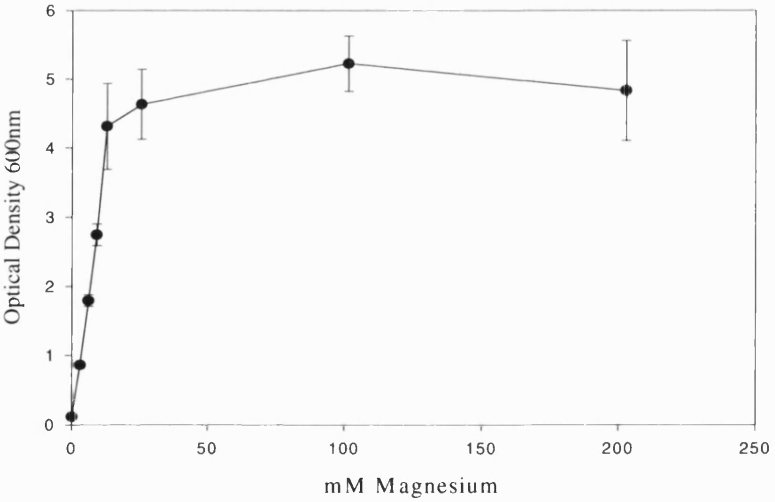


Figure 3.4 – Effect of magnesium concentration in CDM(20) growth medium on planktonic stationary phase density of *B. cereus* ATCC 14579 at 37°C under aeration (mean ± SEM, n=3).

3.3.4 Manganese Limitation

A nutrient limitation control was also completed using manganese. This was to ensure that at the concentration used in CDM(20), it was not imposing a second limitation on the cultures. Manganese is also known to be toxic to *Bacillus* cells at high concentrations, (Cheung *et al.*, 1982b) therefore the concentration used was also studied for toxic, growth-limiting effects. The results, in Figure 3.5, show that the concentration of manganese used (4.48 μM), was deemed appropriate for these studies.

3.3.5 Iron addition

The effect of iron on *B. cereus* growth was also investigated. Since CDM(20) did not contain added iron, studies were completed by adding iron in concentrations between 0 μM and 90 μM (Figure 3.6). CDM(20) has previously been shown to achieve optical densities of 7.0 or greater without any additional iron (see Table 3.1), therefore low-density iron limitation was not possible. Addition of iron did however lead to an increase in final stationary phase cell density when added at 1 μM or greater. A maximal optical density of approximately 12 was also obtained for all concentrations between 1 μM and 90 μM added iron. These data therefore imply either that iron is not an absolute requirement for *B. cereus* growth, but has a beneficial property, or there is sufficient contaminating iron (glassware, chemicals and water) to allow growth of *B. cereus* up to an optical density ~7, but not beyond. These results therefore lead to a revision of the CDM(20) medium, to produce the final medium containing an additional 10 μM iron.

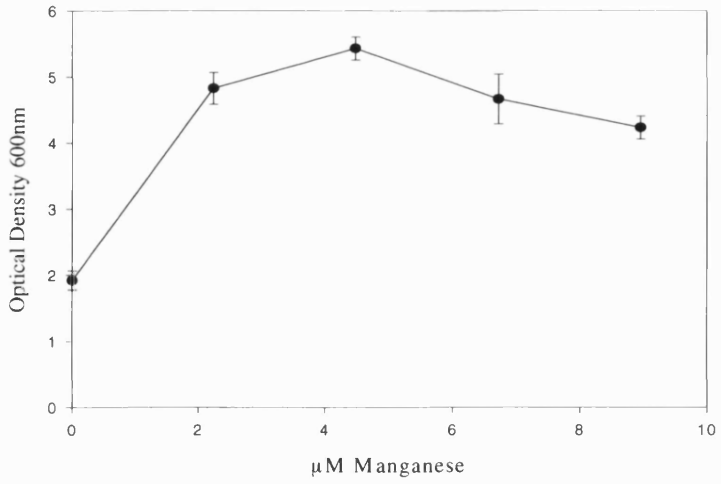


Figure 3.5 – Effect of manganese concentration in CDM(20) growth medium on planktonic stationary phase density of *B. cereus* ATCC 14579 at 37°C under aeration (mean ± SEM, n=3).

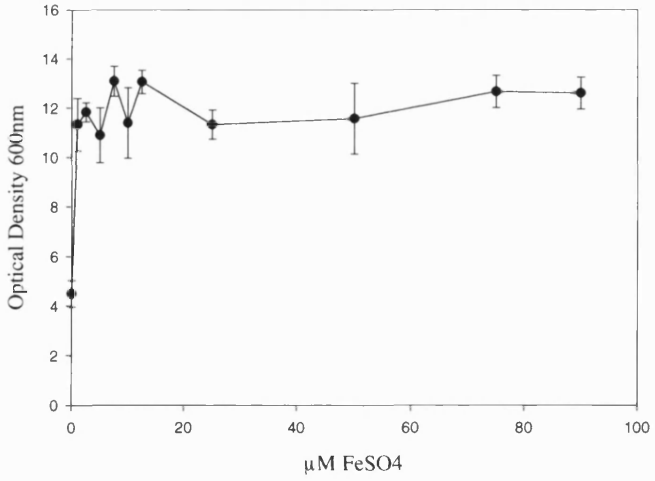


Figure 3.6 – Effect of iron concentration in CDM(20) growth medium on planktonic stationary phase density of *B. cereus* ATCC 14579 at 37°C under aeration (mean ± SEM, n=3).

3.3.6 DTPA Addition

As described previously, low-density iron limitation was problematic due to contaminating iron. DTPA was therefore added to the media as a chelating agent with a high affinity for iron, in order to achieve low-density iron limitation. This was added to CDM(20) without 10 μM iron at concentrations between 0 μM and 90 μM. The resulting data were highly variable, probably due to the variations in the levels of contaminating iron in the cultures (see Figure 3.7); however the general trend shows that at concentrations of 5 μM or greater there is a growth limiting effect of DTPA and at concentrations of 7 μM or greater, growth of *B. cereus* was almost completely inhibited. In order to determine if this growth inhibition was due to toxicity, *B. cereus* cells were cultured in CDM(20) containing 10 μM iron and 10 μM DTPA. Since *B. cereus* was capable of growing in this medium (OD 11.9), it was concluded that addition of DTPA led to iron limitation and DTPA to *B. cereus* is not toxic at 10 μM concentrations. The addition of DTPA therefore achieved low-density iron limitation, however the large degree of variation seen in Figure 3.7 means that this was not a viable method for use in further studies.

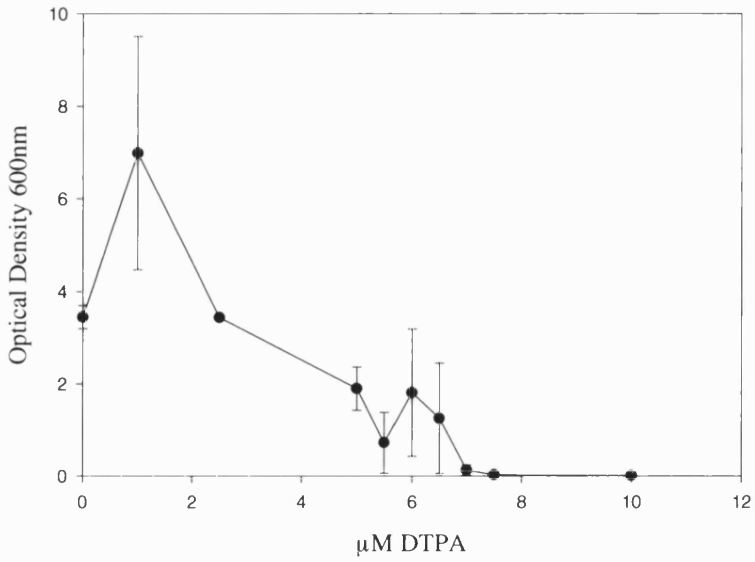


Figure 3.7 – Effect of DTPA concentration in CDM(20) growth medium on planktonic stationary phase density of *B. cereus* ATCC 14579 at 37°C under aeration (mean ± SEM, n=3).

3.4 Planktonic growth curves of nutrient limited cultures

Growth curves were then completed for nutrient limited cultures using the nutrient limiting concentrations described above in section 3.3 (with 41.7 mM glucose) (see Figures 3.8 – 3.11). As expected, all of the growth curves showed that the doubling time similar to the complete CDM(20); however the limitations did have an effect on the lag time of the cultures and the final cell density reached. The lower density was assumed to be due to the imposed limitation and the increase in the lag phase was probably due to the effects of sporulation. It is likely that the overnight nutrient limited cultures used as an inoculum had proceeded further into stationary phase than the complete medium. Therefore, sporulation was more likely to have occurred and the cells would have required an increased amount of time to accustom to the fresh nutrient limited media used in the growth curves.

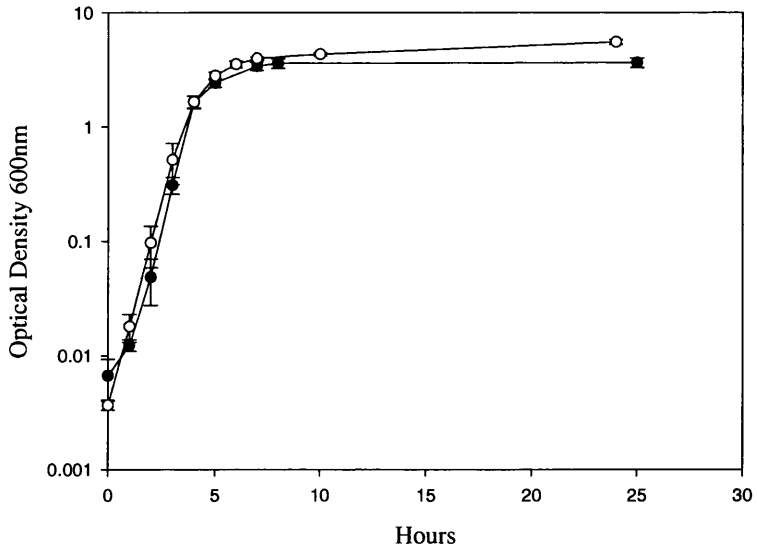


Figure 3.8 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C under aeration in CDM(20) prepared with either 13.9 mM glucose (●) or 41.9 mM glucose (○), (mean ± SEM, n=3).

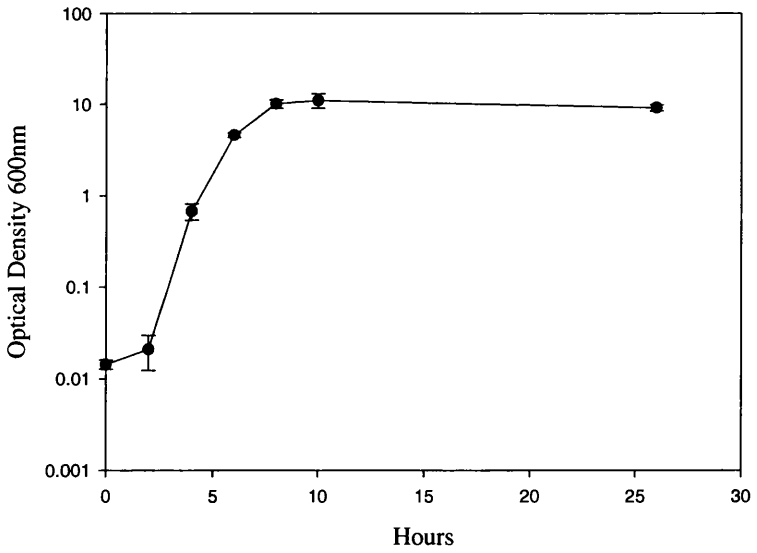


Figure 3.9 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C under aeration in CDM(20) with 10 μM $FeSO_4$ and 41.9 mM glucose, (mean ± SEM, n=3).

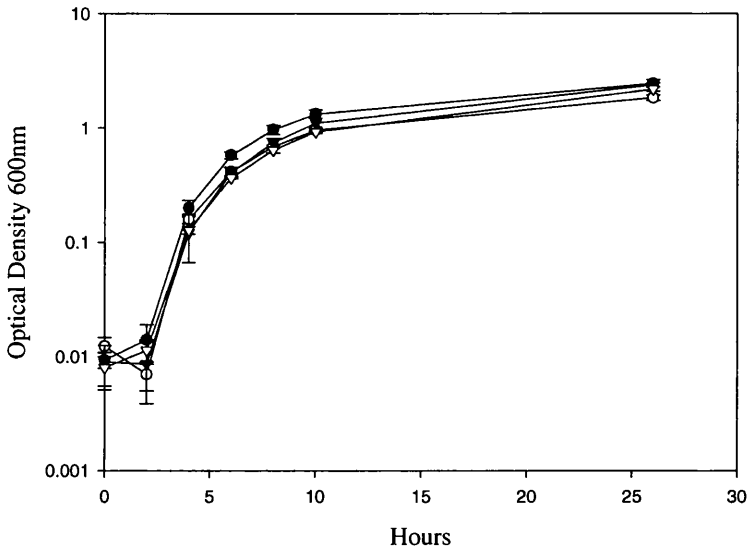


Figure 3.10 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C under aeration in phosphate-limited CDM(20) with (○) or without (●)the addition of 10 μM $FeSO_4$ and magnesium-limited CDM(20) with (▽) or without (▼) the addition of 10 μM $FeSO_4$, (mean ± SEM, n=3).

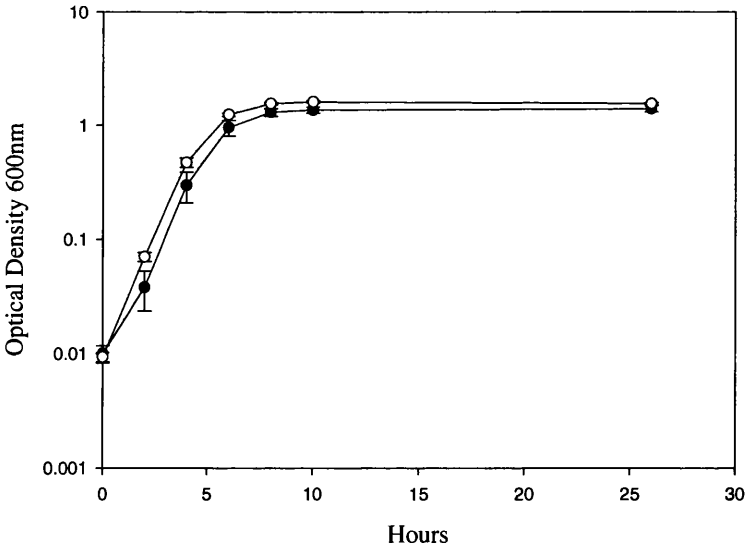


Figure 3.11 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C without aeration in CDM(20) with (○) and without (●) the addition of 10 μM $FeSO_4$, (mean ± SEM, n=3).

3.5 Vegetative cell and spore counts

Figures 3.12 and 3.13 show photographs of *B. cereus* cultures grown in a variety of media for either 24 hours or 48 hours. These figures demonstrate that whilst sporulation events are initiated in the majority of growth conditions studied, very few released spores are visible following 48 hours of growth. In addition, incubation up to 7 days appeared to cause no further increase in released spores. When comparing the appearance of the cells under each of the growth conditions studied, it is clear that the nature of the medium does however have an influence on the gross morphology of the cells.

The cultures grown in CDM under aeration appeared as single rods or short chains containing visible phase bright spores. These cells appeared similar at both 24 hours and 48 hours, implying that any sporulation processes undertaken were either proceeding extremely slowly, or more likely, the *B. cereus* spores formed could not be released from the mother cell in the conditions studied. The addition of iron to CDM(20) did not greatly affect the appearance of the *B. cereus* rods.

Cultures grown statically, with an imposed oxygen limitation, were also similar at the 24 and 48 hour time points. The size and shape of the cells did not appear to change with static incubation when compared with the shaken cultures and addition of iron to the culture did not influence the gross morphology of the cells.

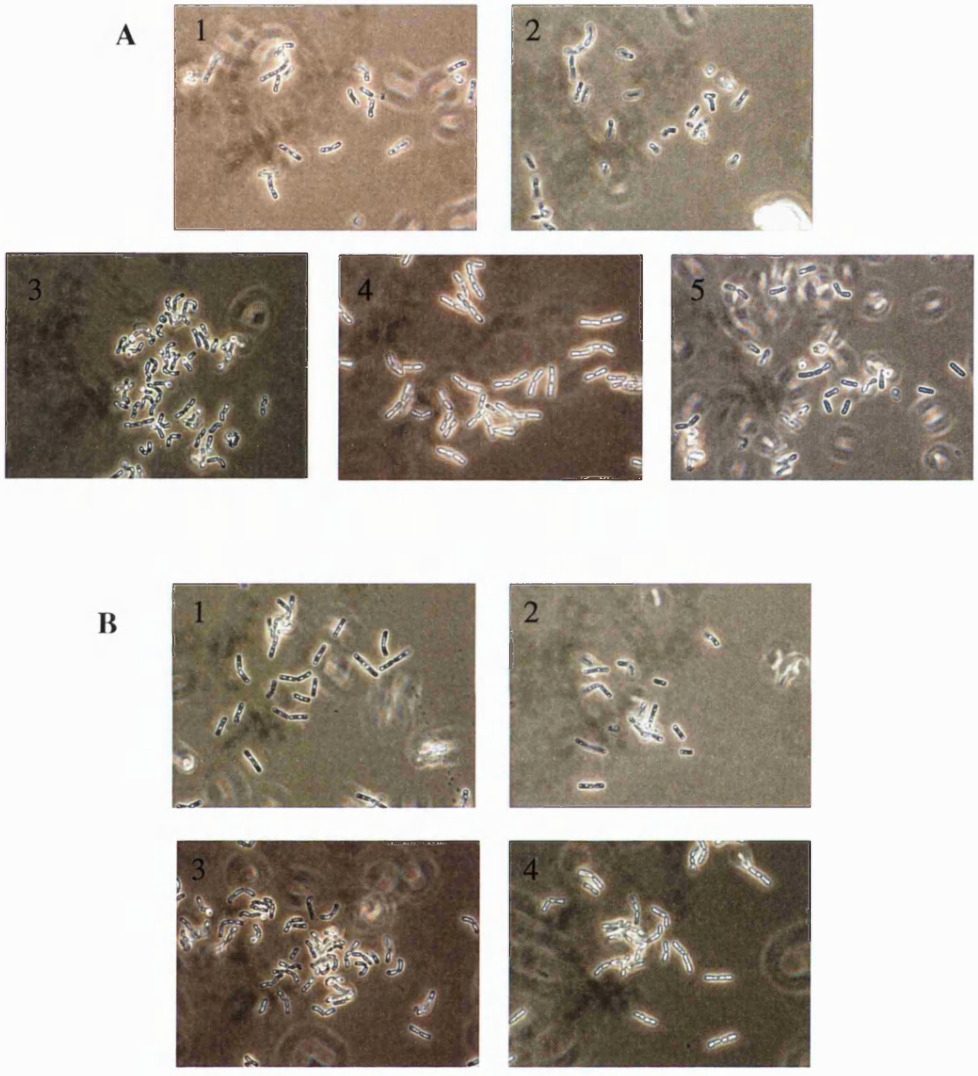
The most noticeable differences in cellular appearance were under phosphate and magnesium-limited conditions. As with the previous conditions, the morphology of the cells was similar at 24 and 48 hours and the addition of iron to the media led to no gross changes in morphology for either the phosphate or magnesium-limited cells. The influence of magnesium limitation was clear, as the cells grown in magnesium-limiting conditions appeared to contain extremely clear phase bright spores and the short chains appeared to be more 'curled' when compared to the other growth conditions.

The influence of phosphate limitation was vastly different to magnesium limitation. The phosphate-limited cells formed longer chains and although the cells appeared phase bright there was no clear spore present in any of the cells and brightness appeared uniform across the whole cell.

Photograph A5 in Figures 3.12 and 3.13 shows the growth of *B. cereus* in LB after 24 and 48 hours, respectively. The cells in these photos, again, appear similar to those seen in CDM(20) and no released spores can be seen at either of the time points. This therefore implies that the lack of released spores seen with the previous conditions is not due to the minimal media used, as no release spores are present in the complex media either. These data therefore lead to the conclusions either that the *B. cereus* strain used is unable to form released spores, or the growth of the strain at 37°C in the media described inhibits the release of spores.

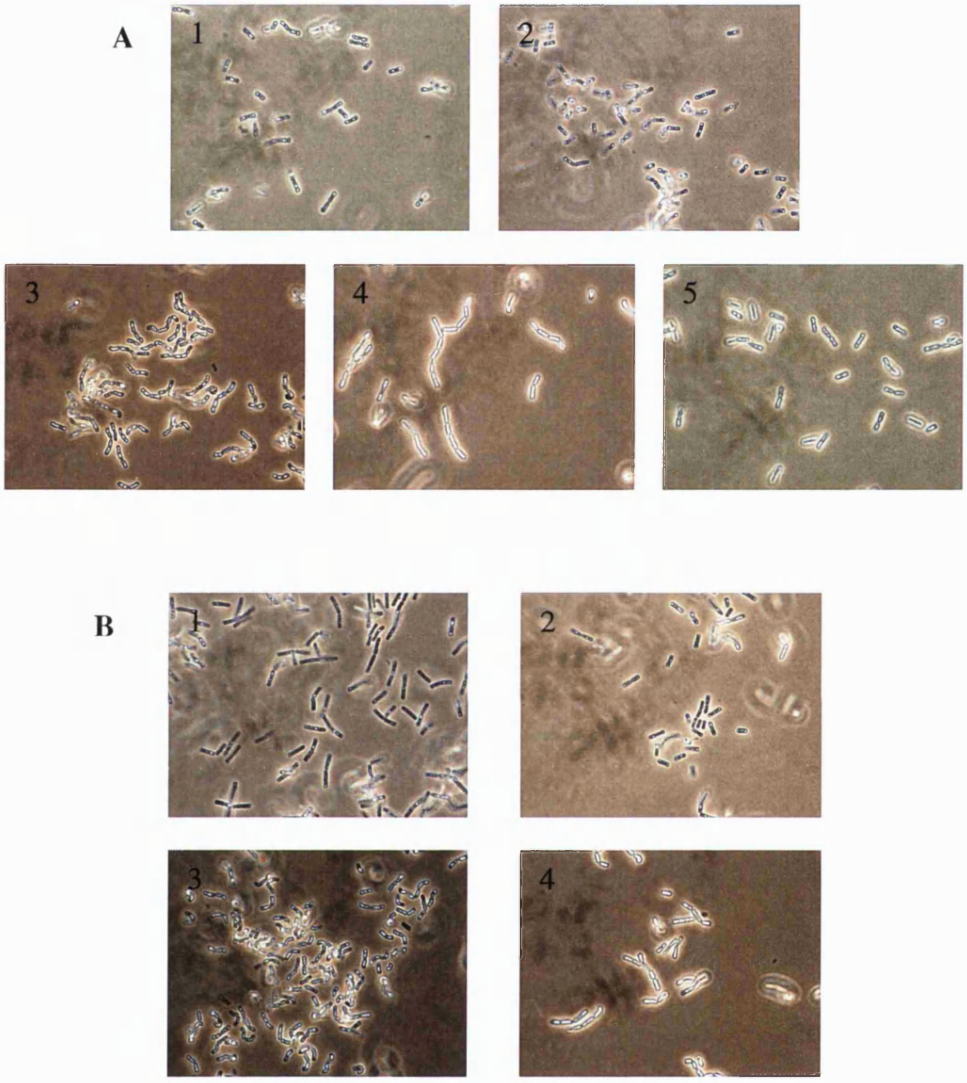
In addition to the photographs, spore counts were also attempted for each of the nutrient limitations studied. These counts were, however, problematic due to the lack of released spores and the subjective assessment of which vegetative cells had initiated the sporulation processes. The problems were enhanced with the use of a haemocytometer, since the depth of the counting chamber allowed the rotation of cells during the process of counting, causing cells that initially looked like released spores to appear to be vegetative cells with or without internal spores – and vice versa. Therefore, unfortunately accurate spore counts could not be completed with the equipment available.

Figure 3.12 - 24 hour cultures of ATCC 14579 *B. cereus* cells grown under various conditions



B. cereus ATCC 14579 under 60x magnification after 24 hours culture at 37°C in 1 - CDM with aeration, 2 - CDM without aeration, 3 – magnesium-limited CDM with aeration, 4 – phosphate-limited CDM with aeration and 5 - LB with aeration. Cultures were incubated either A) alone or B) with the addition of 10 μM $FeSO_4$.

Figure 3.13 - 48 hour cultures of *B. cereus* ATCC 14579 cells grown under various conditions



B. cereus ATCC 14579 under 60x magnification after 24 hours culture at 37°C in 1 - CDM with aeration, 2 - CDM without aeration, 3 – magnesium-limited CDM with aeration, 4 – phosphate-limited CDM with aeration and 5 - LB with aeration. Cultures were incubated either A) alone or B) with the addition of 10 μM $FeSO_4$.

3.6 Heat Resistance of *B. cereus*

Heat kill experiments were completed at 50°C on *B. cereus* cells grown in a variety of media. The temperature of 50°C was chosen since previously reported heat resistance experiments using *B. cereus* ATCC 14579 had also used 50°C (Periago *et al.*, 2002). Figure 3.14 shows the survival curve of cells that had been grown overnight in LB. This figure demonstrates an initial lag in killing for approximately 30 minutes, followed by a gradual decline over a further 90 minutes, with a final 5 log reduction in cell numbers. When these data are compared with Figure 3.15, where cells were previously grown in CDM(20) with addition of iron, at 37°C, it can be seen that although the final reduction is similar, the CDM(20) (with additional iron) grown cells were more susceptible at the earlier time points ($p=0.008$, $z=-2.666$).

Additionally, since in the previously study of *B. cereus* heat resistance, experiments were completed with cells cultures grown at 30°C instead of 37°C (as used in this study), control heat kill assays were also completed with cultures incubated at 30°C. Comparisons of the CDM(20) (with additional iron) cells grown at 30°C and 37°C (Figure 3.15) showed that growth temperature had no influence on the heat resistance of *B. cereus* ($p=0.069$, $z=-1.820$) and the results seen at 30°C were similar to those reported previously (Periago *et al.*, 2002).

Figure 3.16 demonstrates the effect of heat treatment on cells grown in CDM(20) with and without excess iron. These data show that iron limitation has a dramatic influence on the ability of cells to survive at extreme temperatures ($p=0.028$, $z=-2.197$), since the cells grown under iron limitation are much more susceptible to heat kill in the initial 10 minutes of the treatment compared with the CDM(20) (with additional iron). The overall reduction in the iron-limited viable cell number is again similar at approximately 5 logs, however in this case the reduction is complete after 75 minutes in comparison to 120 minutes for CDM(20) with additional iron.

The influence of aeration and oxygen availability on *B. cereus* cell survival was also investigated (Figure 3.17). *B. cereus* cells were grown overnight statically in either CDM(20) or CDM(20) with added iron. When the CDM with additional iron cells were then incubated at 50°C it was found that although the final levels of cell death were similar to the aerated conditions (5 log reduction) (see Figure 3.16), the statically incubated cultures were more susceptible to heat in the initial 30 minutes ($p=0.008$, $z=-2.666$); However, when looking at the iron-limited CDM(20) cultures, the static cultures showed significantly greater heat resistance than the shaken cultures ($p=0.028$, $z=-2.192$), implying that cells grown under the double limitation (oxygen and iron) have an increased heat resistance compared with cells grown under iron limitation alone. Furthermore, comparisons of the two static cultures (iron limited and iron-plentiful) show similar heat kill results for both conditions ($p=0.722$, $z=-0.356$). Taken together, these data therefore imply that cells grown under limitation (oxygen or iron) are more susceptible to heat than unlimited cells; however cells limited by either oxygen or oxygen and iron are still more resistant than cells grown under iron limitation alone, it is therefore feasible that a second limitation (oxygen) reduces the effect of the first limitation (iron).

Heat kill experiments were also completed on magnesium and phosphate-limited cultures, with and without the addition of iron. The results for the magnesium-limited cultures (Figure 3.18) were similar to the results seen with the CDM(20) static cultures with a reasonably rapid decline in cell numbers to an overall 5 log decrease after 120 minutes. Comparisons between the magnesium-limited cultures and the CDM (with additional iron) cultures, showed that magnesium-limitation leads to significantly lower toxin levels ($p=0.008$, $z=-2.666$). When comparing the magnesium-limited data with and without the addition of iron, statistical analysis reveals a significant difference between the two samples ($p=0.013$, $z=-2.497$), with the double limitation (magnesium and iron) being more resistant than the single magnesium limitation. This therefore implies that the introduction of a second limitation (iron) negates the some of the effects of the first limitation (magnesium).

The most surprising results were seen with phosphate-limited cells (Figure 3.19). When these cells were treated at 50°C there was a rapid decline in viable counts for the initial 15 minutes, however after this point the numbers of viable cells levelled off and no more heat kill was apparent. The final decline in cell numbers after 120 minutes was only 2-3 logs and therefore the phosphate-limited cells were more capable of surviving at 50°C than the *B. cereus* cells grown in any of the other media. Also, in line with magnesium limitation, the results show that the introduction of a second limitation (iron) leads to the cells being more initially more resistant to heat kill than the cells grown under phosphate limitation alone ($p=0.005$, $z=-2.803$).

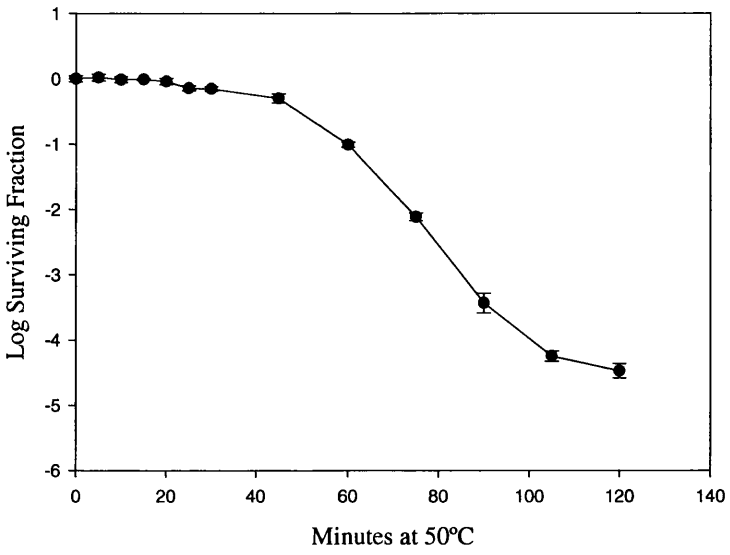


Figure 3.14 - Susceptibility to heat at 50°C of planktonic stationary phase *B. cereus* ATCC 14579 cells grown at 37°C under aeration in LB, (mean ± SEM, n=3).

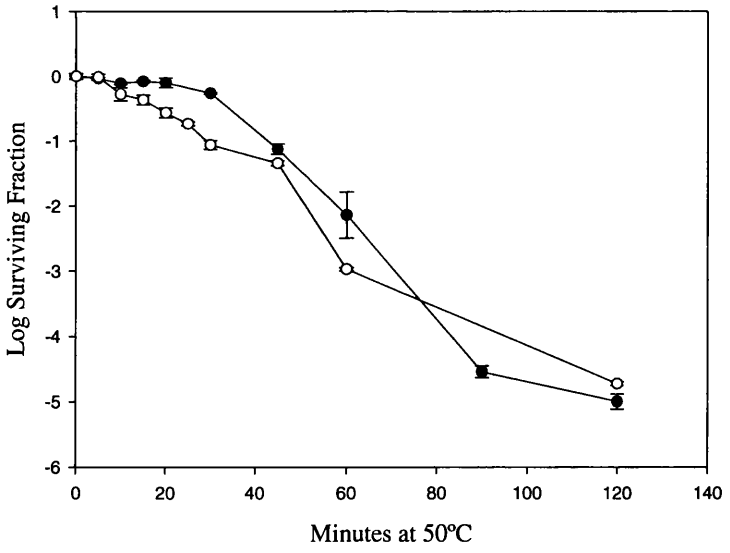


Figure 3.15 - Susceptibility to heat at 50°C of planktonic stationary phase *B. cereus* ATCC 14579 cells grown under aeration in CDM(20) with the addition of 10 µM $FeSO_4$ at 37°C (●) or 30°C (○), (mean ± SEM, n=3).

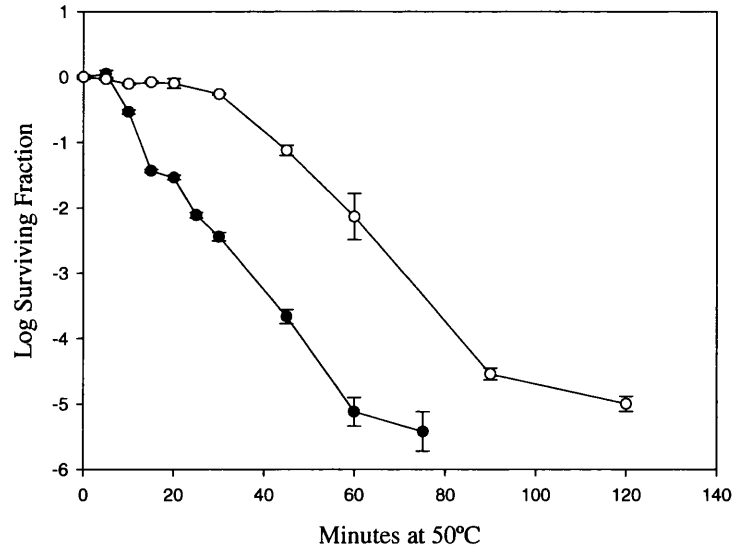


Figure 3.16 - Susceptibility to heat at 50°C of planktonic stationary phase *B. cereus* ATCC 14579 cells grown at 37°C under aeration in CDM(20) with (○) and without (●) the addition of 10 μM $FeSO_4$, (mean ± SEM, n=3).

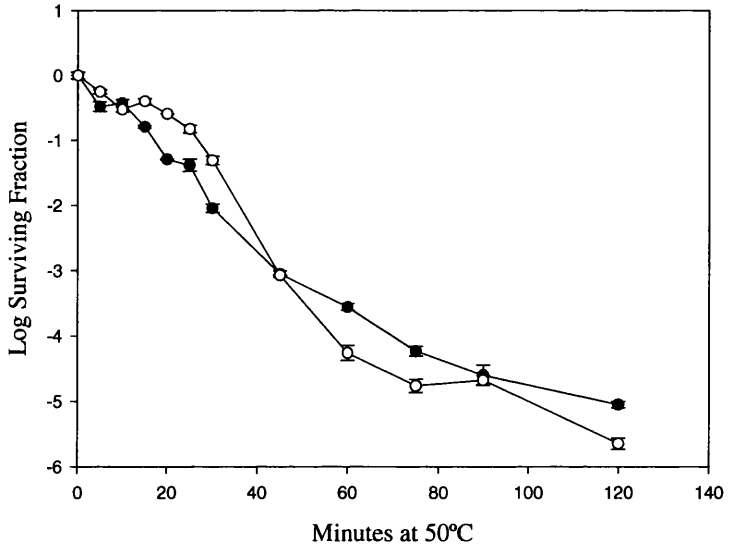


Figure 3.17 - Susceptibility to heat at 50°C of planktonic stationary phase *B. cereus* ATCC 14579 cells grown statically at 37°C in CDM(20) with (○) and without (●) the addition of 10 μM $FeSO_4$, (mean ± SEM, n=3).

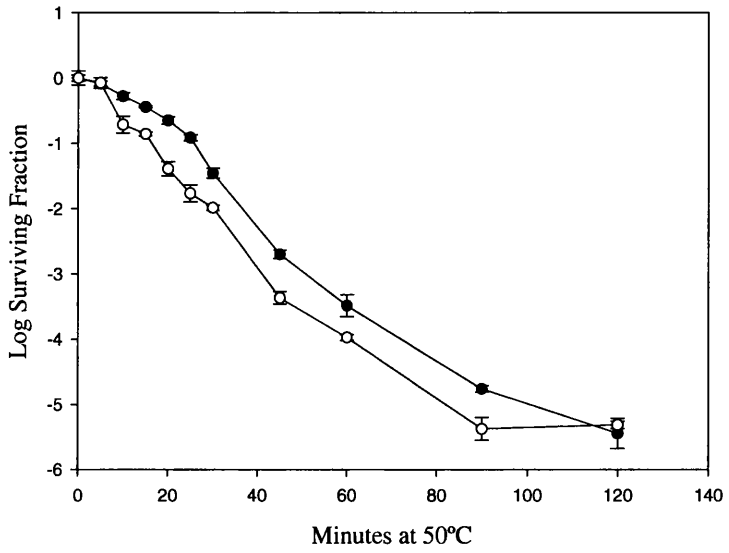


Figure 3.18 - Susceptibility to heat at 50°C of planktonic stationary phase *B. cereus* ATCC 14579 cells grown at 37°C under aeration in magnesium-limited CDM(20) with (○) or without (●) the addition of 10 μM $FeSO_4$, (mean ± SEM, n=3).

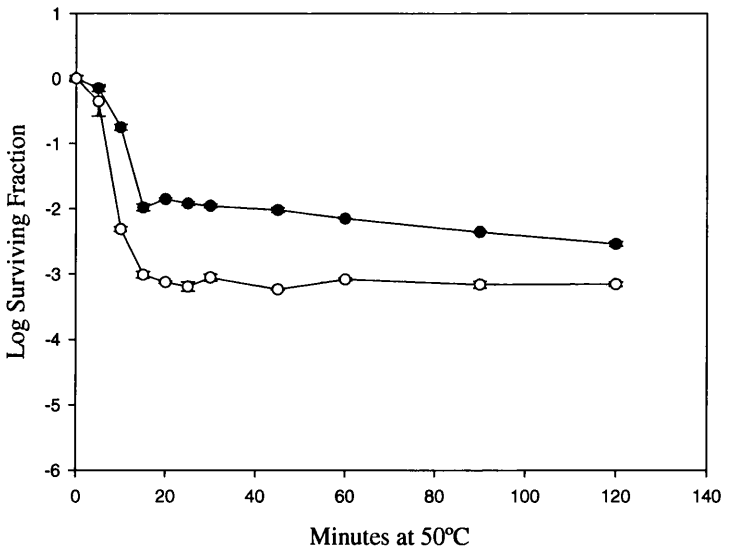


Figure 3.19 - Susceptibility to heat at 50°C of planktonic stationary phase *B. cereus* ATCC 14579 cells grown at 37°C under aeration in phosphate-limited CDM(20) with (○) or without (●) the addition of 10 μM $FeSO_4$, (mean ± SEM, n=3).

3.7 Discussion

3.7.1 CDM for *B. cereus*

A number of chemically defined media for *Bacillus* growth, including *B. cereus*, have been described (Agata *et al.*, 1999; de Vries *et al.*, 2004; Leitch and Collier, 1996; Nakata, 1964; Ristroph and Ivins, 1983). However, these media have only been used in a small number of studies and the majority of works completed on these organisms have used complex media. Unfortunately, the use of complex media leads to heterogeneous growth and sporulation conditions. Therefore this study has derived a chemically defined medium for *B. cereus* growth allowing defined nutrient limitations to be imposed on the cultures.

3.7.2 Amino Acid Requirements

The aims of this study were to investigate the influence of the *B. anthracis* vaccine medium on toxin production and cell physiology, therefore the CDM derived in this study was based on the vaccine medium. However, since the UK *B. anthracis* vaccine medium is not a chemically defined medium due to the addition of casamino acids and charcoal, the casamino acids were supplemented for a mixture of L-amino acids. This substitution allows the completion of nutrient limitation studies and has also been reported to lead to an increase in toxin and virulence factor production (Ristroph and Ivins, 1983).

A screen was therefore completed to determine the amino acid requirements of *B. cereus* ATCC 14579 growth, in order to obtain a CDM for *B. cereus*. The results seen were similar to previous studies investigating *B. cereus* amino acid requirements. These studies have shown that *B. cereus* requires amino acids for growth (White, 1972) and studies with *B. cereus* NC7401 have shown that, as with *B. cereus* ATCC 14579, L-leucine, L-valine and L-threonine are essential (Agata *et al.*, 1999). The similarity between the requirements of *B. cereus* NC7401 and ATCC 14579 therefore suggest similar amino acid requirements across the whole *B. cereus* species; however other studies with number of other *B. cereus* strains have shown a wide variety of amino acid requirements, showing that this is not the case (White, 1972).

3.7.3 *B. cereus* starvation response

Although the effects of starvation on *B. subtilis* are well documented, little genomic data currently exist on the stress response of *B. cereus* and no reports are available on the general stress response formed by *B. cereus* when placed under nutrient limiting conditions. The development of the CDM(20) medium in this study has therefore enabled *B. cereus* to be studied under a range of nutrient limiting conditions including phosphate limitation, magnesium limitation, and high density oxygen, iron and glucose limitations.

As described earlier (see section 1.6.2), studies with *B. subtitlis* have shown that σ^B is activated in response to nutrient limitation (Zhang and Haldenwang, 2003). However, it has been shown that only a limiting activation of σ^B occurs when *B. cereus* cells are depleted in ATP levels, therefore implying that σ^B has no regulatory role in the starvation response of *B. cereus* (van Schaik *et al.*, 2004a).

The results seen for *B. cereus* grown under a variety of nutrient limiting conditions in this study, show that the cells are capable of surviving and growing, hence some form of stress response must occur. If this response is not due to σ^B, it is possible that nutrient limitation of *B. cereus* cells triggers a response regulated by a further alternative sigma factor, a number of which are involved in the sporulation processes of *B. cereus*. It is therefore possible that the nutrient limitation may act as a trigger for sporulation of *B. cereus* and the stress response formed by *B. cereus* is regulated alongside sporulation.

3.7.4 *B. cereus* sporulation

As described earlier (see section 1.6.4), studies into the gross physiology of bacterial sporulation, including numerous *Bacillus* species, have shown that the whether cells sporulate and the bacterial properties of any spores produced, are greatly influenced by the growth conditions of the bacteria (Evans *et al.*, 1997; Gonzalez *et al.*, 1999; Melly *et al.*, 2002). Unfortunately, most of the studies completed with *B. cereus* in this area have used complex media, meaning that the effects of specific nutrient

limitations on sporulation have not been investigated. However, since this study has developed a CDM for *B. cereus*, it has been possible to grow cells under a variety of nutrient limitations and view their sporulation after both 24 hours and 48 hours.

The results from this study have revealed very few spores are fully released, due to the lysis of the mother cell, in any of the media used, including complex media. One explanation of these data is that the *B. cereus* spores are not fully released in the strain (ATCC 14579) used, this is however unlikely due to reports of pure spore preparations using this strain in other studies (de Vries *et al.*, 2004). An alternative explanation for the lack of mother cell lysis may also be due to the culture conditions used, i.e. growth at 37°C. It has been reported that the sporulation process is completed after approximately 7 hours at 37°C with *B. subtilis* (Piggot and Hilbert, 2004); however the genomic differences between *B. cereus* and *B. subtilis*, including differences in their sporulation genes (de Vries *et al.*, 2004) may mean that full sporulation of *B. cereus* does not occur under the same conditions.

Although this study had showed very few released spores in the *B. cereus* cultures, it was clear that sporulation processes had been initiated in all of the growth conditions studied. In addition, it could also be seen that the gross morphology of the cells varied under different nutrient limitations. These changes in sporulation properties have also been previously reported with other *Bacillus* species. A study investigating the effects of nutrient limitation on *B. thuringiensis* sporulation found that although nutrient limitation did not interfere with the processes of sporulation, the character and the yield of the spores produced varied depending on the limitation (Sakharova *et al.*, 1984). Studies with *B. megaterium* have also shown that different nutrient limitations led to variations in growth and sporulation. Sporulation was found to occur in all cultures except those solely limited by potassium, manganese or magnesium. In addition, when spores were produced, the properties of the spores varied in terms of germination, heat resistance and spore volume, depending on the nutrient limitation (Brown and Hodges, 1974). Similar results have also been found with *B.*

stearothermophilus. Studies with this bacterium have found that the nature of the nutrient limitation influences the ability of spores to germinate (Cheung *et al.*, 1982a).

These changes in cell morphology can be attributed to two factors. It is possible that the limiting nutrient is required by the cell in order for the sporulation processes to continue. However this is unlikely in the case *B. thuringiensis* and possibly *B. cereus*, since the sporulation process of *B. thuringiensis* was reported to be unaffected by nutrient limitation (Sakharova *et al.*, 1984). Therefore the changes in seen cellular appearance are more likely to be due to variations in the expression of the sporulation genes and hence their regulators.

3.7.5 Heat Resistance

The development of a CDM for *B. cereus* in this study, has also allowed the completion of heat kill assays on *B. cereus* cells grown under a variety of nutrient and oxygen limitations and temperatures. The results from these assays showed vast differences in the resistance of *B. cereus* cells grown under the varying conditions.

Initially, studies were completed to investigate the influence of growth temperature on *B. cereus* heat resistance. The results showed similar kill curves for cells grown at both 30°C and 37°C, indicating that, in this study, the growth temperature of the *B. cereus* cultures had no influence on the heat resistance of the cells. In contrast to these results, previous studies with *B. cereus* have indicated that the growth temperature does influence the heat resistance of the bacteria (Melly *et al.*, 2002); however, since it has also been reported that the heat resistance of both *Bacillus* spores and vegetative cells is correlated with the maximal growth temperature (Warth, 1978), it is possible that the lack of difference seen with the 30°C and 37°C cultures in this study may reflect the ability of *B. cereus* ATCC 14579 to grow to similar final cell densities under both of the temperatures studied.

The influence of oxygen availability on heat resistance was also investigated in this study, with the results showing that heat shock led to similar final decreases in viable

cell counts with both the oxygen-plentiful and oxygen-limited cultures. This therefore infers that oxygen limitation has no major influence on the overall heat resistance of *B. cereus*. Unfortunately no reports currently exist referring to the effects of oxygen limitation on heat resistance in bacilli; however it has been shown that both oxygen limitation and heat shock up-regulate σ^B in *B. subtilis* leading to a GSR (Eymann and Hecker, 2001). Therefore, it can be predicated that *B. cereus* cells grown under oxygen-limited would up-regulate σ^B, form a GSR and hence be more resistance to further stresses such as heat shock. This trend was not seen in this study, since oxygen limitation in the CDM (with added iron) cultures did not decrease the final amount of heat kill in the cultures and in fact led to the cells being slightly more susceptible at the earlier time points. This can be explained by reports stating that, in *B. subtilis*, a number of the proteins that are up-regulated due to oxygen limitation vary from the heat shock proteins (Hecker and Völker, 1998; Völker *et al.*, 1994), meaning that the GSR response formed due to oxygen limitation may not be effective against heat kill. In addition, currently there are also no reports that oxygen limitation up regulates σ^B in *B. cereus* and therefore the stress response formed due to oxygen limitation may be completely independent of the heat shock stress response. Furthermore, since the CDM (with added iron) oxygen-limited cultures appeared to be initially more susceptible to heat than the oxygen-plentiful cultures, it is also possible that although oxygen limitation led to a GSR, this did not prevent a decrease in cell viability making them more susceptible to further stresses such as heat shock.

Heat shock assays were also completed using nutrient limited cultures. The results showed that the different nutrient limitations led to varying kinetics and levels of heat resistance, with the complete cultures demonstrating the greatest initial heat resistance. When looking at each limitation separately it can be seen that the most susceptible cells were those in CDM without added iron. Previous studies have shown that some of the classical heat shock proteins include molecular chaperones such as DnaK and GroEL. In line with these findings, studies have also shown that these proteins are up-regulated in *B. cereus* after heat shock (Periago *et al.*, 2002). It is known that the chaperone proteins, along with other heat-induced proteins protect the

cells by their roles in protein folding, assembly and repair under stressed and non-stressed conditions. In addition, studies have also found that a number of chaperone proteins, including DnaJ and DnaK interact with iron-sulphur clusters and have a role in the assembly and / or repair of iron-sulphur cluster proteins (Frazzon *et al.,* 2002; Hoff *et al.,* 2000). It is therefore possible that the iron-limited cultures were more susceptible to heat due to the inability of certain heat shock proteins to fully function. In addition since studies with *Mycobactericum smegmatis*, have shown that cells produce the major heat shock proteins (including DnaK and GroEL) under both iron-plentiful and iron-limiting conditions (Lundrigan *et al.*, 1997), it is feasible that the iron-limited *B. cereus* cultures produced the required heat shock proteins but were still more susceptible to heat shock due to a limited concentration of iron-sulphur clusters in the cells.

The relationship between nutrient limitation and heat resistance has been previously reported for other bacilli. Studies with *B. subtilis* have shown that when cells are grown on media supplemented with varying metal ions, there are differences in the heat resistance of the spores formed (Cazemier *et al.*, 2001). Furthermore, other studies investigating the physiology of *Bacillus* spores formed due to a range of nutrient limitations have shown that nutrient limitation can influence the heat resistance of spores (Brown and Hodges, 1974; Mazas *et al.*, 1995). Therefore it can be seen, that unlike oxygen limitation and temperature, nutrient limitation does vastly influence the heat resistance of the cells.

3.7.6 Stress adaptation

As mentioned previously (see section 1.6.2), studies with *B. cereus* have shown that when cells are exposed to a mild treatments (such as temperatures of 42°C, 4% ethanol, acid stress, 2.5% NaCl or hydrogen peroxide), an adaptation process is initiated that increases resistance to lethal stresses such as an acid stress of pH 4.6 or temperatures of 50°C (Browne and Dowds, 2001; Browne and Dowds, 2002; Periago *et al.*, 2002; van Schaik *et al.*, 2004a). Therefore, the use of nutrient and oxygen-

limiting conditions in this study has provided an opportunity to see if a stress adaptation occurs in *B. cereus* by nutrient limitation prior to heat kill.

Since the data from these heat kill studies have shown that, in general, the nutrient limited cultures are less resistant to heat treatment than cultures grown in complete media, it can be inferred that nutrient limitation of *B. cereus* does not allow the cells to become more resistant to further stresses in the same way as mild heat, ethanol, or acid treatments do. These results are not surprising, since heat resistance and stress adaptation of *B. cereus* is believed to be regulated by the alternative sigma factor σ^B (van Schaik *et al.*, 2004a). Studies have shown that heat shock from 30°C to 42°C causes a 20.1 fold activation of σ^B and in addition, osmotic shock and ethanol exposure have also been shown to lead to σ^B activation; however, as stated earlier, ATP depletion has been shown to not significantly activate σ^B, and hence nutrient limitation of *B. cereus* is unlikely to up-regulate σ^B. Therefore, although the nutrient limitation of *B. cereus* probably leads to a GSR, there is no induction of σ^B, meaning that no σ^B regulated adaptation can occur prior to exposure to lethal heat temperatures. In contrast to earlier observations, it should however be noted that phosphate limitation did led to an increase in heat resistance of *B. cereus,* therefore it is possible that phosphate limitation involves a different stress response than the other nutrient limitations that may involve σ^B, although this has not yet been shown.

3.7.7 Conclusions

To conclude, a medium has been developed that allows studies into the effects of nutrient limitation on the gross physiology of *B. cereus*. It has been shown that although complete sporulation is unable to occur in any of the nutrient limited media studied, the process is initiated in all of the limitations studied and the morphology of the cells varies depending on the growth conditions. Studies investigating the heat resistance of *B. cereus* have shown that, generally nutrient limited cultures are more susceptible than cells grown in complete media. It has been previously implied that nutrient limitation of *B. cereus* does not induce σ^B, and hence nutrient limitation does not allow a stress adaptation prior to heat treatments. The results seen in this study

support these findings, with the exception of phosphate limitation that leads to a greater heat resistance than cells grown under phosphate-plentiful conditions. This therefore implies that phosphate limitation leads to a form of stress adaption that may involve σ^B activation. Overall, these data show that nutrient limitation of *B. cereus* affects the gross physiology of the cells, implying that there may also be a degree of variation in other cellular aspects such as virulence factor production, as discussed in the next chapter.

Chapter 4 - Toxin and protease production under nutrient limitation

4.1 Introduction

As described previously, nutrient limitation has been shown to influence a number of virulence factors in bacteria, including the production of toxins and degrading enzymes (Agata *et al.*, 1999; Ombaka *et al.*, 1983; Ristroph and Ivins, 1983). A number of these studies have been completed investigating the production of *B. cereus* toxins under nutrient limitation and environmental stresses. Studies with the emetic toxin have shown that optimal production occurs at 30°C (Szabo *et al.*, 1991) and no production occurs above 37°C (Finlay *et al.*, 2000). Additional studies have shown that emetic toxin production is vastly enhanced by the addition of glucose to the growth medium and the amino acids; valine, leucine and threonine are a requirement for *B. cereus* growth and toxin production. Data also show that cereulide production is vastly reduced when the growth medium is supplemented with high concentrations of leucine, isoleucine and glutamic acid (Agata *et al.*, 1999), demonstrating that as with other bacilli, the growth medium of *B. cereus* influences toxin production.

Taking these studies into account, it was hypothesised that the introduction of nutrient limiting conditions for both *B. cereus* and *B. anthracis* would lead to changes in the kinetics and yield of the *B. cereus* enterotoxins and *B. anthracis* toxins described in sections 1.5.4 and 1.3.3 respectively. In addition, since it is likely that the UK anthrax medium leads to nutrient limited growth of *B. anthracis* (Personal communication – Mike Hudson, HPA), it is likely that the medium used vastly influences the quantity of toxins produced. The aims of this study were therefore to investigate the effects of nutrient limitation on toxin production in both *B. cereus* and *B. anthracis* in order to see if the nutrient limitations imposed in the vaccine medium are able to enhance the yield of anthrax toxins or are actually hindering maximal toxin production.

4.2 *B. cereus* toxin production

HBL toxin levels were measured in CDM(20) with and without the addition of 10 μM $FeSO_4$ under different nutrient limiting conditions. The toxin levels were determined using the Oxoid *B. cereus* BCET-RPLA enterotoxin test kit. Since no quantitative positive control was available, relative amounts of the enterotoxin were estimated based on a detection limit of 2 ng / ml toxin in media, as stated in the manufacturer's instructions. Unfortunately, since this assay could only give positive and negative results and no quantitative levels of aggregation could be determined, end point titres were required in order to determine the toxin levels in the cultures. The samples were therefore serially diluted using 1 in 2 dilutions with the last positive dilution being used to calculate the toxin levels in the culture. Due to the nature of serial dilutions this method introduced a reasonable degree of error and variation with the higher dilutions, since the actual toxin levels in the samples, although calculated using the last positive titre, could have actually been at any level between the last positive and the first negative result. For example, when a sample was serially diluted and the last positive result was calculated as 64 ng / ml, with the first negative being 128 ng / ml. The result was recorded as 64 ng / ml although the actual levels could have been anywhere in the region of 64 -127 ng / ml toxin.

Figure 4.1A demonstrates the influence of iron on the toxin titres of CDM(20). The kinetic profiles show a rise in toxin levels after 4 hours with a peak between 6 and 10 hours, after which the levels decline to the initial levels. Assays on the toxin levels for CDM(20) with and without addition of $FeSO_4$ showed high toxin levels for *B. cereus* cultures grown in both media and comparisons between the two cultures showed no significant difference between the two cultures ($p=0.138$, $z=1.438$). When comparing the toxin profiles of the oxygen-limited cultures (Figure 4.1B) with and without added iron, it is clear that the addition of iron also had no influence on the toxin levels ($p=0.5888$, $z=-5.42$). The toxin levels seen in the CDM(20) with iron cultures are similar with both the static and shaken cultures, inferring that the static incubation of *B. cereus* does not have any influence on toxin production in the complete medium ($p=0.456$, $z=-0.730$).

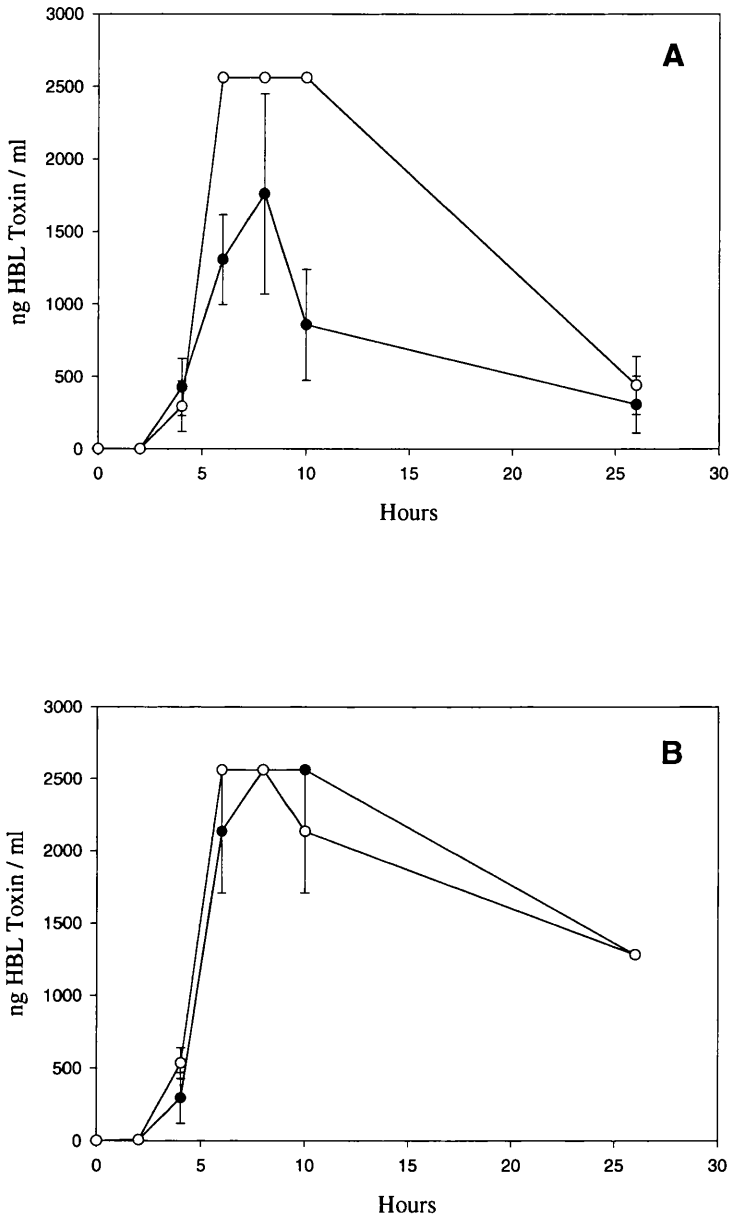


Figure 4.1 – Kinetics of toxin production for *B. cereus* ATCC 14579 grown at 37°C in CDM(20) either **A** – under aeration, or **B** –statically, with (○) or without (●) the addition of 10 μM $FeSO_4$ (mean ± SEM, n=3).

Figure 4.2 demonstrates the influence of magnesium and phosphate limitation on the toxin profiles. It can be seen that the addition of iron has no influence over the initial nutrient limitation, since the profiles for both magnesium and phosphate-limited cultures do not change with the addition of iron (phosphate; $p>0.999$, $z=0$, magnesium; $p=0.456$, $z=-7.30$). Comparisons with the CDM(20) plus iron cultures show that the toxin levels seen under the phosphate and magnesium limitations are vastly lower than the CDM(20) plus iron toxin levels (shown in Figure 4.1) (phosphate; $p=0.043$, $z=-2.023$, magnesium; $p=0.043$, $z=-2.023$). In addition, it can also be seen that the kinetics of toxin production are altered under phosphate and magnesium limitations. The magnesium-limited cultures (Figure 4.2A) have the most dramatic peak in toxin production, the levels begin to rise after 4 hrs and peak at 6 hrs at approximately 60 ng toxin / ml, after which there is a sharp decline concluding with undetectable toxin levels after 26 hours. The phosphate-limited cultures (Figure 4.2B) demonstrate the lowest levels of toxin seen under any of the limitations. There is a slight rise in levels after 4-6 hours to approximately 15-20 ng toxin / ml followed to a gentle decline to steady state levels of around 10 ng / ml.

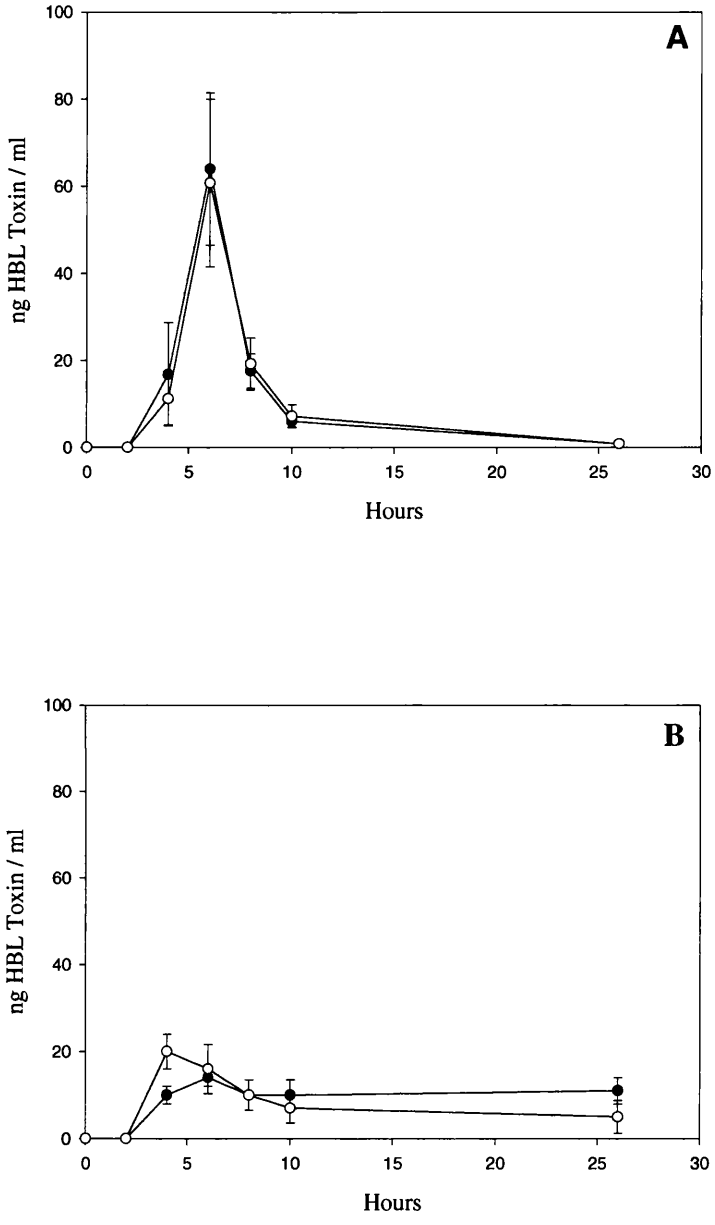


Figure 4.2 – Kinetics of toxin production for *B. cereus* ATCC 14579 grown at 37°C under aeration in **A** - magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) either with (○) or without (●) the addition of 10 μM $FeSO_4$ (mean ± SEM, n=3).

4.3 *B. cereus* protease production

The previous section clearly shows differences in toxin levels and kinetics when *B. cereus* is grown under varying nutrient limiting conditions. These variations can be explained by differences in the amount of toxin produced in each culture, or may be explained by the production of proteases degrading the toxin. In addition the influence of proteases may also explain the sharp declines seen in toxin levels seen under magnesium-limited conditions.

For these reasons the protease levels in the varying nutrient limited cultures were also measured. The original protease assays were completed using an assay developed by S. Twining, (Twining, 1984); however high background levels and variations in repeat samples due to poor stability of a temperamental fluorometer, led to the assays being repeated using a modified version of the Twinning assay as used in the Calbiochem protease assay kit. This kit uses a casein substrate conjugated to FTC. Incubation with protease cleaves the conjugated proteins to release FTC peptides, the remaining FTC-casein is then precipitated and removed and the absorbance of the FTC peptides is measured at 492nm, indicating the protease activity of the sample.

Studies were completed determining the protease levels in *B. cereus* cultures grown under a number of nutrient and oxygen-limiting conditions. Figure 4.3 demonstrates the protease levels seen in cultures grown in CDM either with or without the addition of iron. Panel A shows the protease levels in the cultures grown under aeration, it can be seen that under these conditions there is a notable difference in the protease levels of the iron-plentiful and iron-limited cultures ($p=0.018$, $z=-2.371$). When *B. cereus* is grown under aeration in the presence of iron, maximal protease levels are seen after 6-10 hours with absorbance levels reaching approximately 0.3. *B. cereus* cells grown under equivalent conditions without the addition of excess iron, showed a slightly later peak at 10 hours with absorbance only reaching approximately 0.18. The iron-plentiful culture displayed only a slight reduction in protease levels after 26 hours of culture, whilst the protease levels in the iron-limited culture declined back to baseline levels at the final time point recorded.

The influence of oxygen limitation on protease levels is show in panel B. This figure shows similar protease levels for both the iron-limited and the iron-plentiful cultures when grown statically ($p=0.499$, $z=-6.76$). This therefore implies that an additional iron limitation has no effect on protease levels when cells are already limited by oxygen. The kinetics of the statically grown cultures show a peak in protease levels after 6 hours with an absorbance of 0.23-0.24. Similarly to the iron-limited aerated cultures, the protease levels then show a marked decline, with the final 26 hour time point only recording baseline levels.

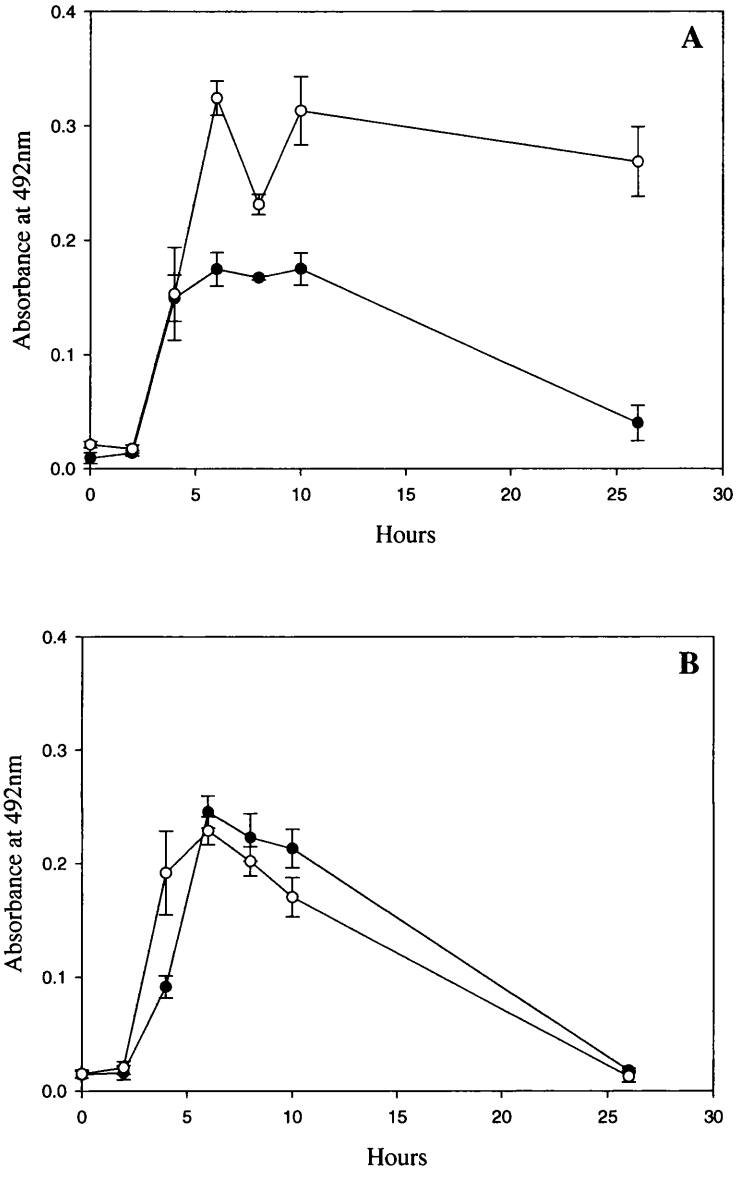


Figure 4.3 – Kinetics of protease production for *B. cereus* ATCC 14579 grown at 37°C in CDM(20) either **A** – under aeration, or **B** –statically, with (○) or without (●) the addition of 10 μM $FeSO_4$ (mean ± SEM, n=3).

The influence of phosphate and magnesium limitation on protease levels was also investigated (Figure 4.4). Panel A shows the protease levels of the *B. cereus* cultures grown under a magnesium limitation. The cultures were grown either with or with out the presence of excess iron and the results show, that as with oxygen limitation, the introduction of a second iron limitation has no influence on the protease levels beyond the first limitation (magnesium) ($p=0.063$, $z=-1.859$). When comparing the iron-plentiful magnesium-limited culture with the CDM (with added iron) culture, (Figure 4.3) a marked difference can be seen ($p=0.028$, $z=-2.197$). The magnesium-limited cultures showed a much lower peak in protease levels with the highest absorbance readings only reaching 0.18, the kinetics of the protease production were also slightly different with the peak occurring later at 10 hours. Similarly to the CDM culture, the magnesium-limited cultures continued to produce proteases throughout the entire time course, with the final 26 hour time points showing similar protease levels to the 10 hour peak, inferring a sustained peak in protease production or a peak at a time point between 10 and 26 hours where no samples were taken.

Panel B shows the influence of phosphate limitation on protease levels. Unlike oxygen limitation and similarly to magnesium limitation, the protease levels of the phosphate-limited cultures did vary with an additional iron limitation ($p=0.018$, $z=-2.366$), with the phosphate-limited iron-plentiful cultures showing higher protease levels than the iron-limited phosphate-limited cultures. The kinetics of both the phosphate-limited cultures (with and without excess iron) were however reasonably similar. Both cultures showed a peak in protease levels at 10 hours and reasonably high proteases levels were then sustained until the 26 hour time point. It should however be noted that the absorbance of the phosphate-limited iron-plentiful culture did drop from approximately 0.25 to 0.18 in the later stages, where as the absorbance of the iron-limited phosphate-limited cultures remained approximately level. When the protease levels of the phosphate-limited iron-plentiful culture are compared with the CDM (with added iron) culture (Figure 4.3), it can be seen that the phosphate-limited culture contains lower maximal protease levels (phosphate = OD 0.245, CDM (with iron) = OD 0.324) and the kinetics of protease production have also shifted.

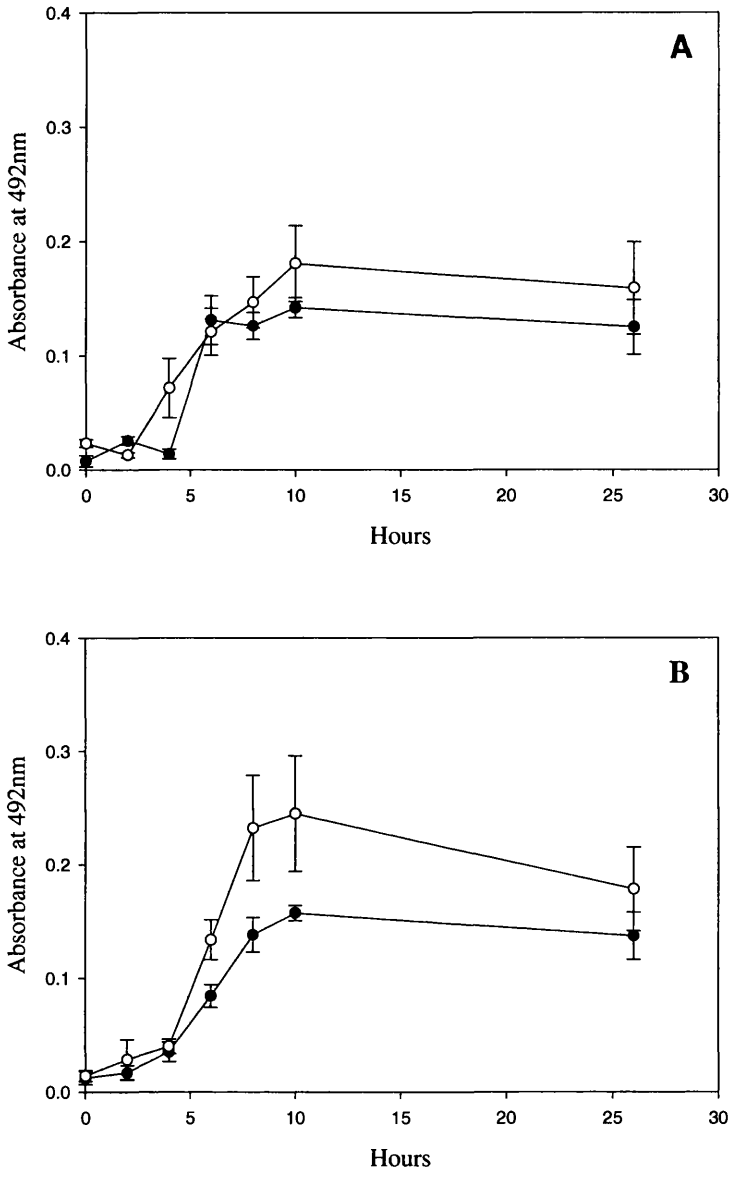


Figure 4.4 – Kinetics of protease production for *B. cereus* ATCC 14579 grown at 37°C under aeration in **A** – magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) either with (○) or without (●) the addition of 10 μM $FeSO_4$ (mean ± SEM, n=3).

4.4 Inhibition of *B. cereus* proteases using protease inhibitors

Although the results have shown that *B. cereus* produces different protease levels and kinetics under varying nutrient limiting conditions, whether these factors actually influence toxin production still remained unclear. For this reason, protease inhibitors were also added to the *B. cereus* cultures and protease assays were then completed in order to show that the inhibitors added were specific for blocking the *B. cereus* produced proteases. Toxin assays were then completed on the protease inhibited cultures in order to understand if the proteases had an influence on the toxin kinetics under each of the nutrient limitations.

In order to add the protease inhibitors to the cultures, an inhibitor had to be chosen that allowed maximal *B. cereus* growth whilst inhibiting protease production. The initial protease inhibitor selected was PMSF. A range of concentrations was then added to the *B. cereus* cultures. Unfortunately at all the concentrations of PMSF used, there was an inhibition of bacterial growth leading to much lower stationary phase optical densities, ruling out comparative studies between cultures with and without the addition of inhibitor. Despite this, a protease assay was completed (using the original Twining assay) on the *B. cereus* cultures with or without 1 mM PMSF. The assay results showed vast differences in the protease levels of each culture, with the PMSF positive culture not recording any protease levels above base line. These data therefore show that although PMSF is a specific protease inhibitor for *B. cereus* proteases, its addition to *B. cereus* cultures is toxic to cells and therefore PMSF is inappropriate for these protease inhibition studies.

For this reason, PMSF was substituted for protease inhibitor cocktails (PICs). The first PIC selected contained, Pepstaitin A, Leupeptin, Antipain and Aprotinin. These were added at a 10 μg / ml concentration (or 2 μg / ml for Pepstatin A) as recommended for tissue culture studies. There was no, or little, influence on the growth of *B. cereus*; however protease assays also revealed that the cocktail used was not specific for the *B. cereus* proteases since no detectable differences in protease levels were found in samples with or without the PIC.

The PIC was therefore changed for a bacterial specific PIC from Sigma. This cocktail contained; AEBSF, EDTA, Bestatin, Pepstatin A and E-64. These components are believed to inhibit serine proteases, metalloproteases, aminopeptideases, acid proteases and cysteine proteases respectively. A titration of the PIC was then completed in order to establish the maximal non-toxic volume that could be added to the *B. cereus* cultures. This titration clearly showed that the addition of PIC inhibits *B. cereus* growth when added in volumes of 10 µl / 20 ml culture or greater; however final cell density is unaffected when PIC is added at 4 µl / 20 ml culture or lower.

In order to full demonstrate that 4 µl or less PIC / 20 ml culture did not effect *B. cereus* growth, growth curves were then completed in CDM(20) for cultures either with or without the addition of 4 µl PIC (Figure 4.5 – A). These graphs show that although similar final densities are obtained in both cultures, the growth rate is vastly diminished in the PIC positive culture implying that the addition of 4 µl PIC influences *B. cereus* growth. For this reason growth curves were also completed on CDM(20) cultures containing 1 µl, 1.5 µl and 2 µl PIC (Figure 4.5 - B). These growth curves show similar growth rates and final cell densities for all of the cultures implying that when PIC is added to cultures at volumes of 2 µl or lower / 20 ml CDM, there is no effect on the growth kinetics of *B. cereus* and hence these volumes are suitable for further studies.

Growth curves were then also completed for both phosphate and magnesium-limited cultures. These growth curves were completed on cultures that either contained 0 µl, 1 µl, 1.5 µl or 2 µl PIC. Similarly to the CDM(20) cultures, the addition of PIC at these volumes to the phosphate and magnesium-limited media did not influence the growth rate or stationary phase density of the *B. cereus* cultures (see Figure 4.6).

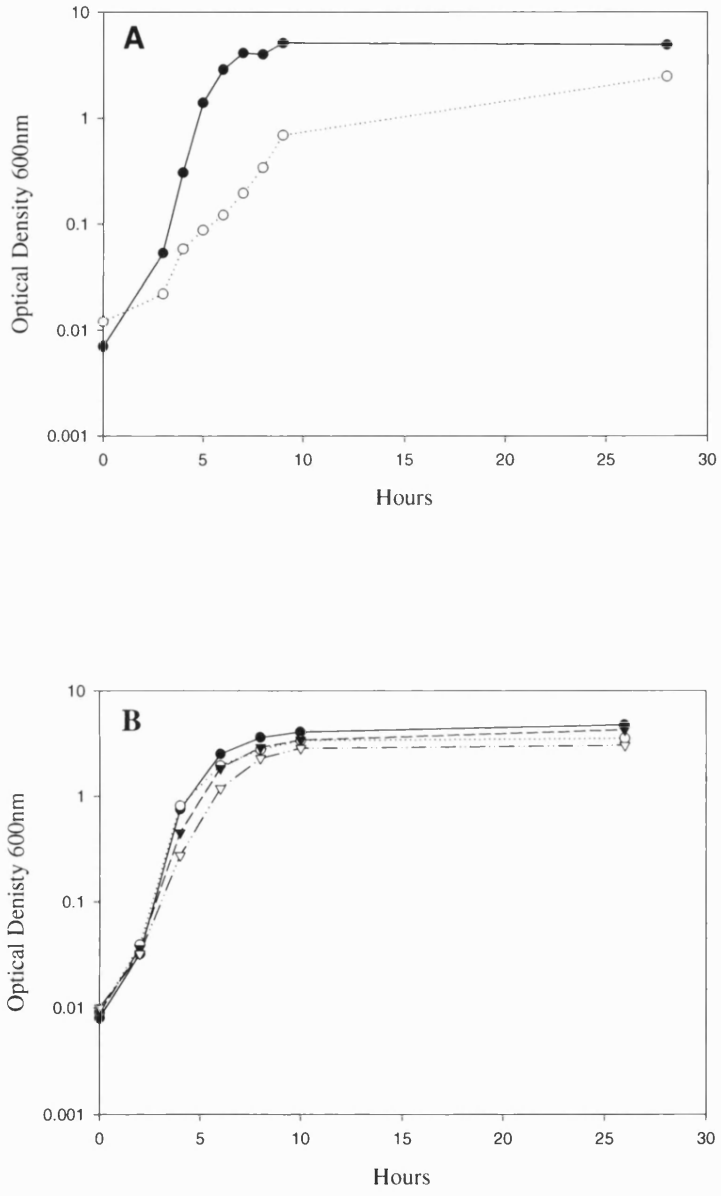


Figure 4.5 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C under aeration in CDM(20) **Panel A**; without PIC addition (●) or 4 µl PIC (○). **Panel B**; without PIC addition (●), 1 µl PIC (○), 1.5 µl PIC (▼) and 2 µl PIC (▽) (n=1).

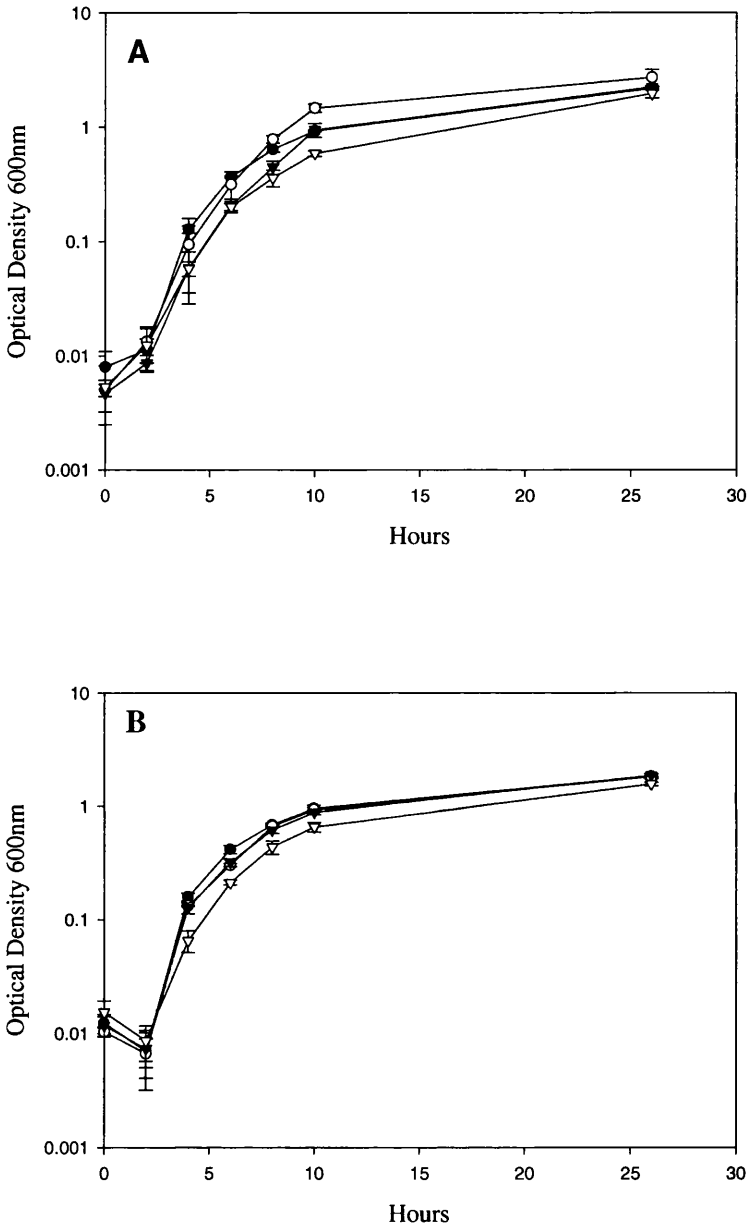


Figure 4.6 – Planktonic growth of *B. cereus* ATCC 14579 at 37°C under aeration in **A** – magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) either without PIC (●), 1 µl PIC (○), 1.5 µl PIC (▼), or 2 µl PIC (▽), (mean ± SEM, n=3).

Having completed growth curves for a number of nutrient limitations containing varying amounts of PIC, protease and toxin assays were completed on the samples taken from the growth curves. Figure 4.7 shows protease assays for CDM(20) *B. cereus* cultures grown either with or without the addition of protease inhibitors. This figure shows an overall significant difference between the samples (Friedman Test, $p=0.002$, $\chi_3=12.074$). As expected, the highest protease levels were seen in the CDM(20) cultures that did not contain any PIC. The cultures containing 2 µl PIC shows slightly diminished (but not significant) protease levels ($p=0.075$, $z=-1.782$) and the cultures containing 4 µl show vastly lower protease levels than the 0 µl control ($p=0.018$, $z=-2.371$). These data therefore imply that the PIC inhibits the protease activity of the *B. cereus* cultures in a dose dependant manner. It should however be noted that since the 4 µl PIC cultures also had variations in the growth kinetics, compared to the control, the differences in protease levels may also be attributed to this.

Figure 4.8 demonstrates the influence of PIC on the toxin levels in the *B. cereus* CDM(20) cultures. From the graph it can seen that the same maximal levels were detected in all of the cultures. It can be seen that there is a slight shift in the kinetics of toxin production with the 4 µl PIC sample peaking slightly later than the 0 µl and 2 µl PIC samples, however, overall these data imply that the addition of PIC to the CDM(20) cultures has little influence on the toxin production of the samples (Friedman Test, $p=0.282$, $\chi_3=0.233$). This therefore implies that the production of proteases in the CDM(20) cultures does not influence the toxin levels of the samples.

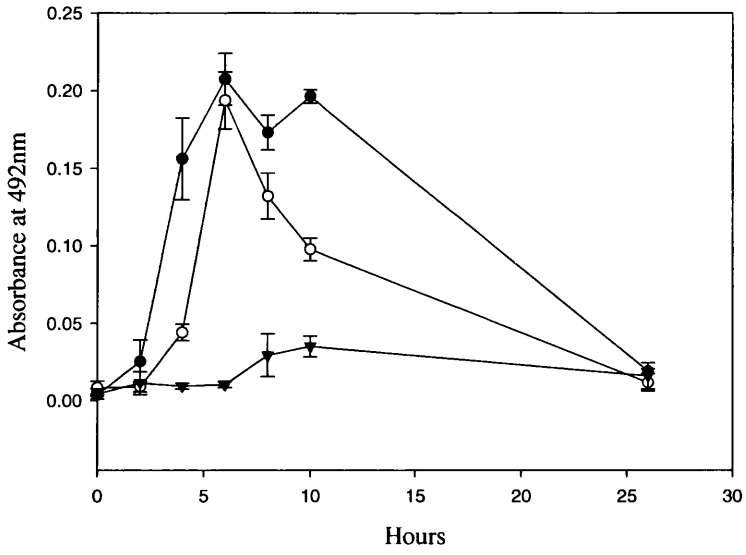


Figure 4.7 – Kinetics of protease production for *B. cereus* ATCC 14579 grown at 37°C in CDM(20) with 0 µl PIC (●), 2 µl PIC (○) and 4 µl PIC (▼), (mean ± SEM, n=3).

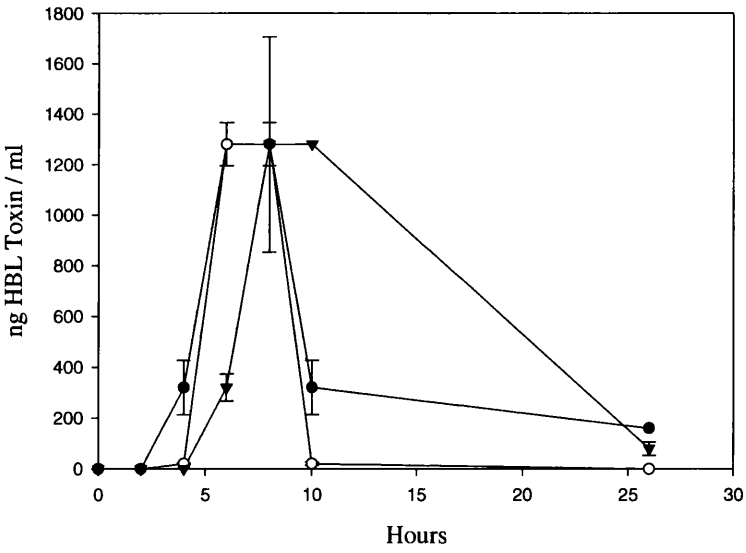


Figure 4.8 – Kinetics of toxin production for *B. cereus* ATCC 14579 grown at 37°C in CDM(20) with 0 µl PIC (●), 2 µl PIC (○) and 4 µl PIC (▼), (mean ± SEM, n=3).

The influence of PIC was also investigated on phosphate-limited CDM(20) and magnesium-limited CDM(20). As stated earlier, the addition of PIC in volumes up to 2 µl had no apparent effect on the growth kinetics of the limited cultures and therefore studies were completed investigating the effects of up to 2 µl PIC on the protease and toxin levels of the cultures.

Figure 4.9 demonstrates the influence of PIC on the protease levels of the cultures, a show a significant overall difference between the samples (phosphate p=0.001, χ_3=16.543; magnesium p=0.001, χ_3=16.739). In a similar way to the CDM(20) cultures, there are lower protease levels in the phosphate and magnesium-limited cultures that also contain 2 µl PIC (phosphate p=0.018, z=-2.366; magnesium p=0.018, z=-2.366). The addition of 1.5 µl PIC also appeared to lower the protease levels in the magnesium-limited CDM(20) cultures (p=0.027, z=-2.207) as well as decreasing the maximal peak in the phosphate-limited cultures (p=0.018, z=-2.371). The addition of 1 µl PIC to the magnesium-limited cultures also slightly decreased the protease levels (p=0.043, z=-2.028) however had no effect on the phosphate-limited cultures (p=0.128, z=-1.524) in terms of maximal production or the kinetics of production. This therefore implies that this dose is insufficient to inhibit the *B. cereus* proteases produced in the phosphate-limited cultures.

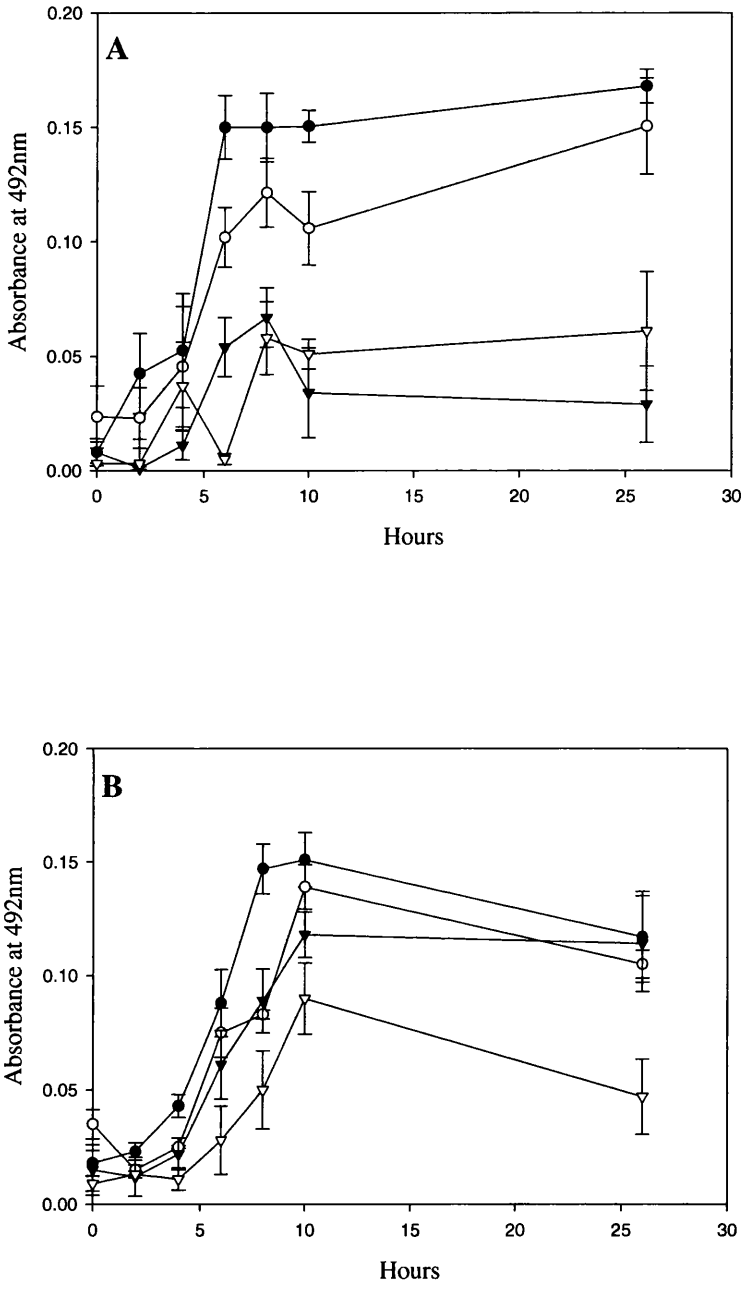


Figure 4.9 – Kinetics of protease production for *B. cereus* ATCC 14579 grown at 37°C under aeration in **A** – magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) with 0 µl PIC (●), 1 µl PIC (○), 1.5 µl PIC (▼) and 2 µl PIC (▽), (mean ± SEM, n=3).

In addition, Figure 4.10 shows the toxin levels of the *B. cereus* phosphate and magnesium-limited cultures with and without the addition of PIC. A significant increase in toxin levels is seen with the addition of PIC (phosphate p=0.017, χ_3=10.188; magnesium p=0.017, χ_3=10.20). The results for the magnesium limited cultures show that whilst 1 µl PIC (p=0.043, z=--2.023) and 1.5 µl PIC (p=0.043, z=-2.023) lead to maximal levels in the *B. cereus* toxin, the addition of 2 µl PIC leads to no significant increase in toxin levels in comparison with the 0 µl control (p=0.138, z=-1.483). This therefore infers that the 2 µl dose has an inhibitory or toxic effect on the culture. The results for the phosphate-limited cultures show that all of the doses of PIC (1 µl p=0.042, z=-2.032; 1.5 µl p=0.042, z=-2.032 and 2 µl p=0.042, z=-2.032) lead to a significant increase in toxin levels. However, the maximal levels of the protease-inhibited phosphate-limited cultures (128 ng / ml toxin) were still, vastly lower than the protease-inhibited magnesium-limited cultures, where maximal yields were approximately ten fold higher with the 1 µl and 1.5 µl PIC doses and nearly 4 fold higher with the 2 µl dose.

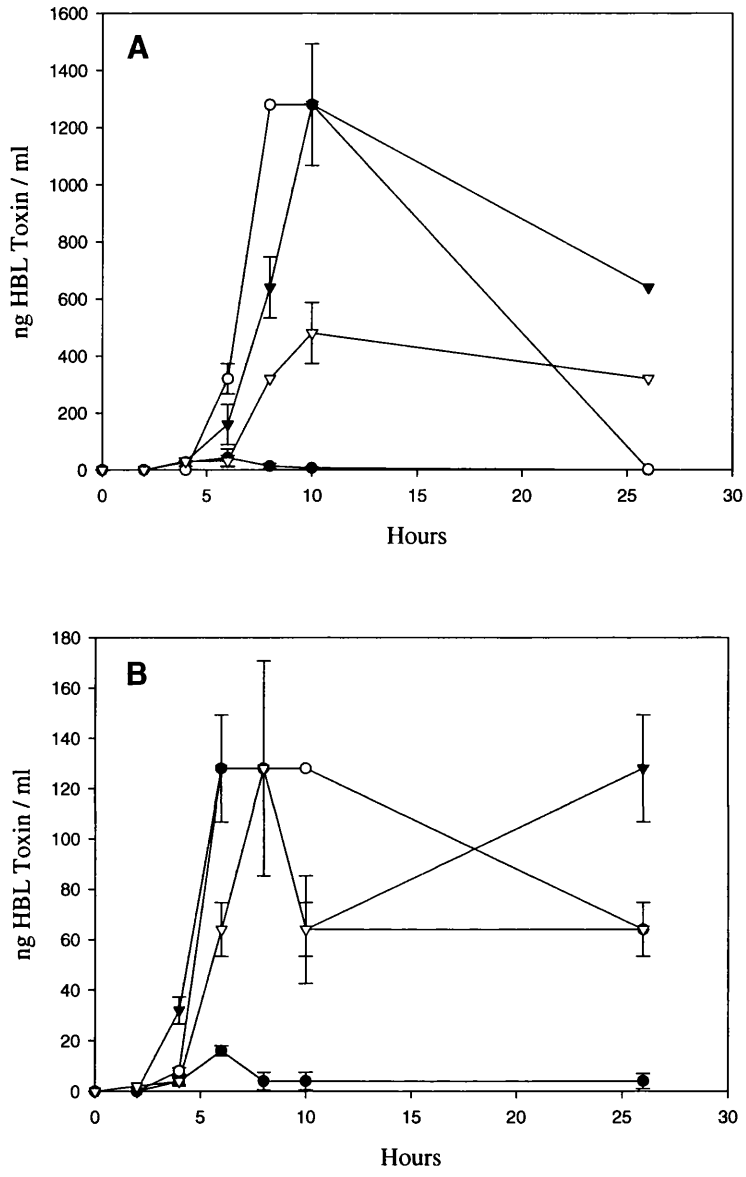


Figure 4.10 – Kinetics of toxin production for *B. cereus* ATCC 14579 grown at 37°C under aeration in **A** – magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) with 0 µl PIC (●), 1 µl PIC (○), 1.5 µl PIC (▼) and 2 µl PIC (▽), (mean ± SEM, n=3).

4.5 Inhibition of *B. cereus* proteases using EDTA

Since all of the *B. cereus* proteases previously described have been metalloproteases (see section 1.5.2) and the addition of EDTA to the bacterial PIC mix is believed to inhibit metalloproteases, further studies were initiated investigating the effects of adding EDTA alone to the CDM(20), magnesium-limited CDM(20) and phosphate-limited CDM(20) cultures.

Since EDTA was added to the PIC at a 100 mM concentration, the same concentration of EDTA alone was prepared. A titration was then completed with the 100 mM in a similar way as PIC, in order to determine optimal doses of EDTA where there was no inhibition of growth, but inhibition of protease activity. Like PIC, EDTA was found to be completely inhibitory to *B. cereus* growth when added to cultures in 20 µl volumes / 20 mls. EDTA was also found to be slightly inhibitory at volumes greater than 4 µl / 20 mls, but had no effect on final cell density when added in 2 µl / 20 ml volumes. Since the PIC had been added to cultures in volumes ranging from 1 µl PIC to 4 µl PIC, growth curves were completed with CDM(20) cultures containing similar levels of EDTA.

As shown in Figure 4.11, EDTA affects *B. cereus* growth in a similar way to PIC. Although similar final optical densities occurred with all of the doses studied, the growth rate of the culture containing 4 µl EDTA was lower than the 0 µl EDTA control. The growth rates of the 1 µl and 2 µl EDTA cultures appeared to be reasonably similar to the control, inferring that, as with PIC, the addition of EDTA at these doses has no major inhibitory effects in terms of *B. cereus* growth yield and kinetics.

Similar growth curves were then also completed for the phosphate and magnesium-limited CDM(20) cultures (see Figure 4.12). As can be seen, the addition of 2 µl EDTA to these cultures caused no apparent differences in the growth rate or final yield of the cultures, indicating that at the 2 µl dose there are again no inhibitory effects on *B. cereus* growth.

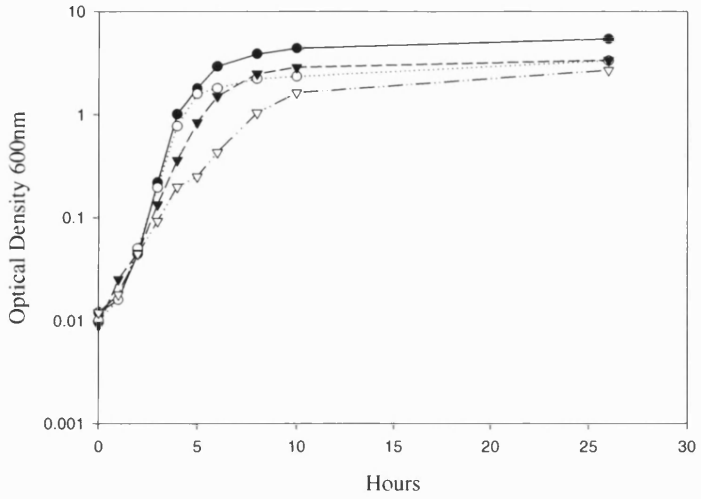


Figure 4.11 – Planktonic growth of *B. cereus* ATCC 14579 at 37ºC under aeration in CDM(20); without EDTA addition (●), 1 µl EDTA (○), 2 µl EDTA (▼) and 4 µl EDTA (▽), (n=1).

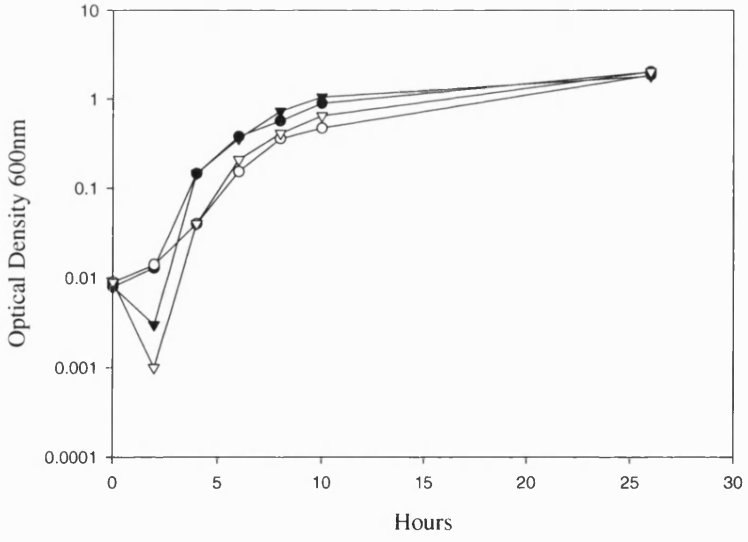


Figure 4.12 – Planktonic growth of *B. cereus* ATCC 14579 at 37ºC under aeration in either magnesium-limited CDM(20) without EDTA (●) or 2 µl EDTA (○) or in phosphate-limited CDM(20) without EDTA (▼) or 2 µl EDTA (○); (▽), (n=1).

Having completed growth curves with and without the addition of EDTA for a number of nutrient limitations, samples were taken in order to complete protease and toxin assays. When investigating the influence of EDTA on the CDM(20) cultures, addition of EDTA appeared to inhibit protease activity in a dose dependant manner (Figure 4.13). The results show a vast decrease in protease levels with the addition of 4 µl EDTA, compared with the 0 µl EDTA control. In addition a slight decrease in the later protease levels was also seen with the addition of 2 µl EDTA. When comparing these results with the protease assays completed on the CDM(20) cultures with and without the addition of PIC (Figure 4.7), it can be seen that the influence of EDTA is similar to PIC. This provisional data therefore infers that the protease inhibition seen with PIC addition is due to the inclusion of EDTA within the cocktail.

Figure 4.14 demonstrates the influence of EDTA on the CDM(20) toxin levels. Similar kinetic profiles can be seen for both the 0 µl and 2 µl EDTA cultures; however, surprisingly there is a decrease in maximal toxin levels with the addition of 2 µl EDTA. Since these data are only provisional (n=1), no conclusions can be drawn, but it is feasible that addition of 2 µl EDTA is not only failing to enhance the toxin levels of the CDM culture, as seen with PIC, but may also be having an inhibitory role.

It should also be noted that the difference in the maximal toxin levels shown is only based on provisional data and due to only one serial dilution. Therefore the actual difference between the amounts of enterotoxin in the two cultures may not be accurately reflected in Figure 4.14. As described in section 4.2, the levels recorded are the last dilution used that gave a positive result. This therefore means that there is the potential of positive variation in either one or both of the 0 µl and 2 µl samples. Hence the difference in toxin levels between these samples may actually be either greater or smaller than the difference depicted.

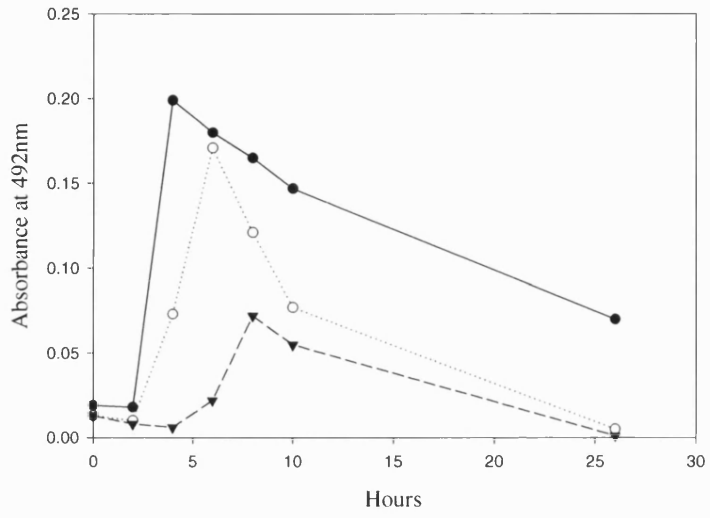


Figure 4.13 – Kinetics of protease production for *B. cereus* ATCC 14579 grown at 37°C under aeration in CDM(20) with 0 µl EDTA (●), 2 µl EDTA (○) and 4 µl EDTA (▼) (n=1).

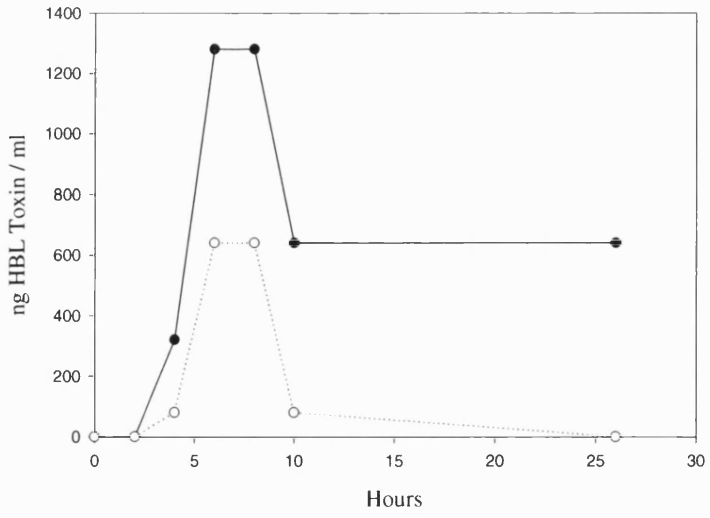
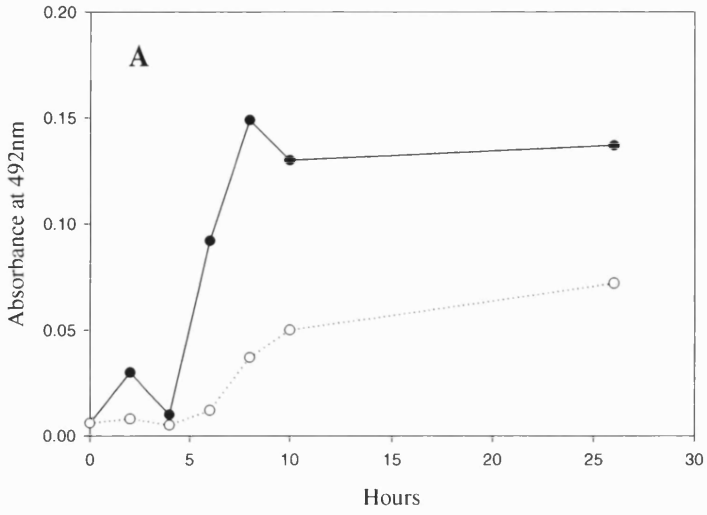


Figure 4.14 – Kinetics of toxin production for *B. cereus* ATCC 14579 grown at 37°C under aeration in CDM(20) with 0 µl EDTA (●) and 2 µl EDTA (○) (n=1).

The protease and toxin levels were also assayed in the magnesium and phosphate-limited cultures that had been prepared with and without the addition of EDTA. The influence of EDTA on the protease levels is shown in Figure 4.15. These graphs indicate that the addition of 2 µl EDTA to the cultures leads to a decrease in protease levels in comparison to the 0 µl controls, inferring that the addition of EDTA to either phosphate-limited or magnesium-limited CDM(20) is capable of limiting the metalloprotease activity of these cultures.

In addition, Figure 4.16 demonstrates the influence of EDTA and metalloprotease inhibition on the toxin levels of the magnesium and phosphate-limited CDM(20) cultures. These provisional results show that the addition of 2 µl EDTA led to an increase in toxin levels of these nutrient limited cultures, with the magnesium-limited cultures showing greater maximal levels than the phosphate-limited cultures.

In the case of the phosphate-limited cultures, the addition of 2 µl EDTA appears to have a similar effect on toxin levels as the addition of 2 µl PIC, (as shown in Figure 4.10). This therefore implies, that in the case of phosphate limitation, the influence of PIC on toxin levels can be explained by the presence of the metalloprotease inhibitor, EDTA. It should however be noted that in the case of the magnesium-limited EDTA-inhibited culture, the toxin levels do not reach the same maximal levels as the magnesium-limited PIC-inhibited cultures (see Figure 4.10), inferring that some other factor in the PIC mix is also capable of increasing the toxin levels.



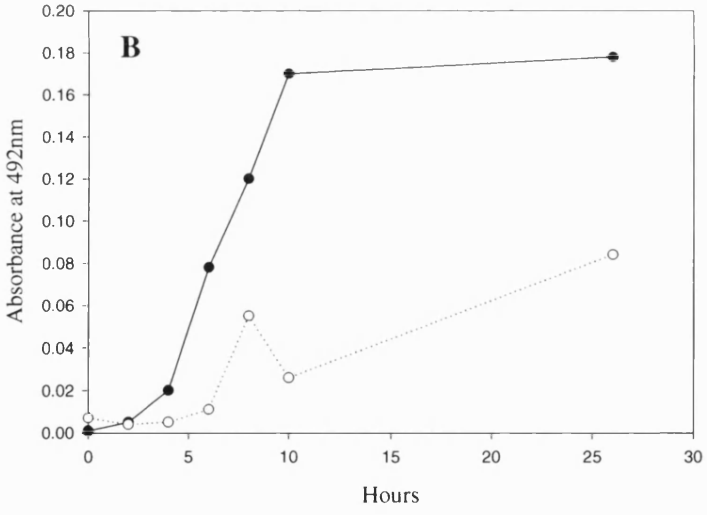


Figure 4.15 – Kinetics of protease production for *B. cereus* ATCC 14579 grown at 37°C under aeration in **A** – magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) with 0 μM EDTA (●), and 10 μM EDTA (○) (n=1).

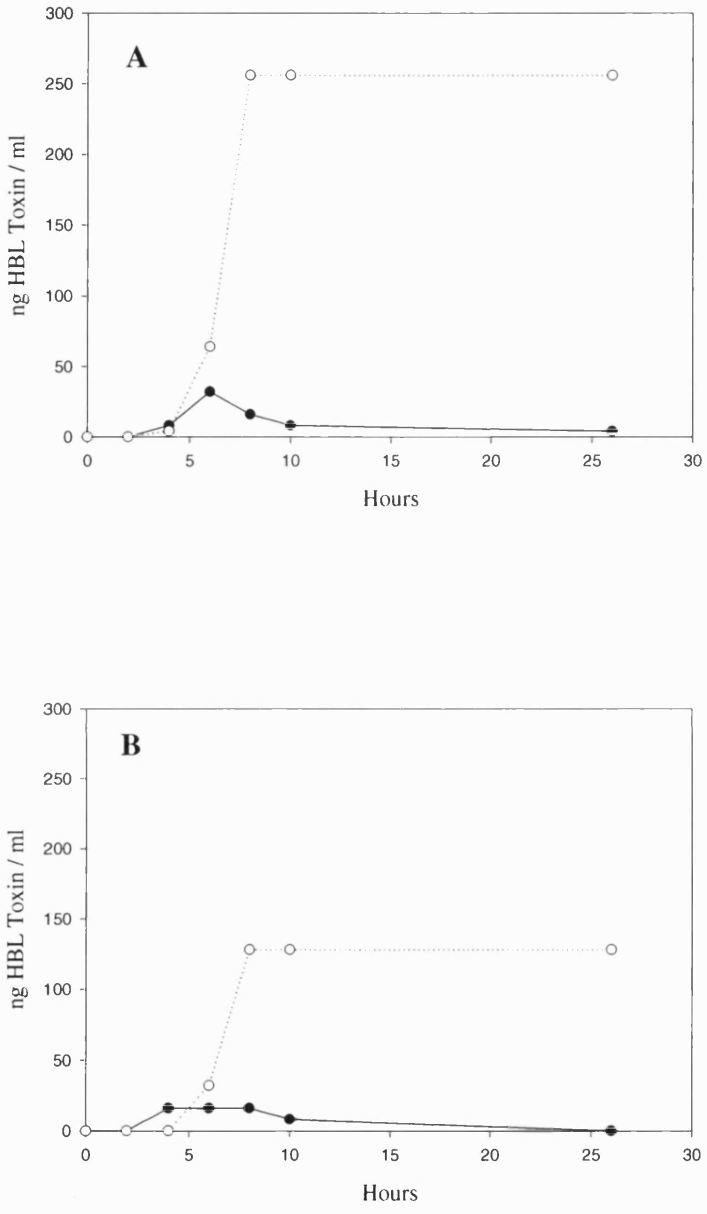


Figure 4.16 – Kinetics of toxin production for *B. cereus* ATCC 14579 grown at 37°C under aeration in **A** – magnesium-limited CDM(20) and **B** – phosphate-limited CDM(20) with 0 μM EDTA (●), and 10 μM EDTA (○) (n=1).

4.6 *B. anthracis* growth and toxin production

Since nutrient limitation was found to influence the toxin production of *B. cereus* cultures, similar work was completed with the *B. anthracis* Sterne strain. This is the strain used in the anthrax vaccine production.

The CDM(20) with additional iron, derived for *B. cereus* was therefore used to complete a *B. anthracis* growth curve, in addition, growth curves were also completed for a number of nutrient limitations including; CDM(20) without iron, phosphate-limited CDM(20) with iron, magnesium-limited CDM(20) with iron and CDM(20) with iron grown statically (oxygen-limited).

These growth curves are shown in Figures 4.17 and 4.18. Unfortunately the cell numbers at the later time points are not completely accurate due to severe clumping of the bacteria, despite additional treatments aimed at re-suspending them, such as vortexing. Despite these problems, it can be seen that *B. anthracis* is capable of growing in all the variations of CDM(20) studied, with the phosphate, magnesium and oxygen-limited cultures achieving lower final optical densities than the complete or iron-limited cultures, implying that a degree of nutrient limitation is occurring.

However, when comparing the optical densities with the *B. cereus* cultures, it can be seen that *B. anthracis* growth occurs to a much lower density with all the nutrient limitations studied than the equivalent *B. cereus* cultures. This strongly infers that although the CDM(20) medium and nutrient limited derivatives, are able to support growth, they are lacking an essential nutrient required in order to obtain high cell densities with *B. anthracis*. It is possible that this element is available in trace amounts through 'glassware' and water contamination, however is not available in sufficient concentrations to allow high levels of growth. If this is the case, it is also therefore possible that the limiting nutrient is also having an influence on the vaccine medium, since growth of *B. anthracis* in the vaccine production also only occurs at low levels.

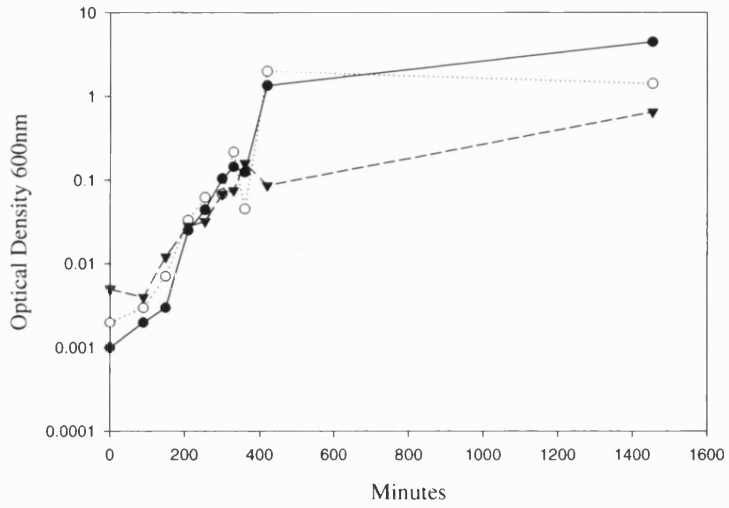


Figure 4.17 – Planktonic growth of *B. anthracis* Sterne at 37°C in CDM(20) with aeration (●), CDM(20) with 10 μM $FeSO_4$, with aeration (○) and CDM(20) with 10 μM $FeSO_4$, under static incubation (▼), (n=1).

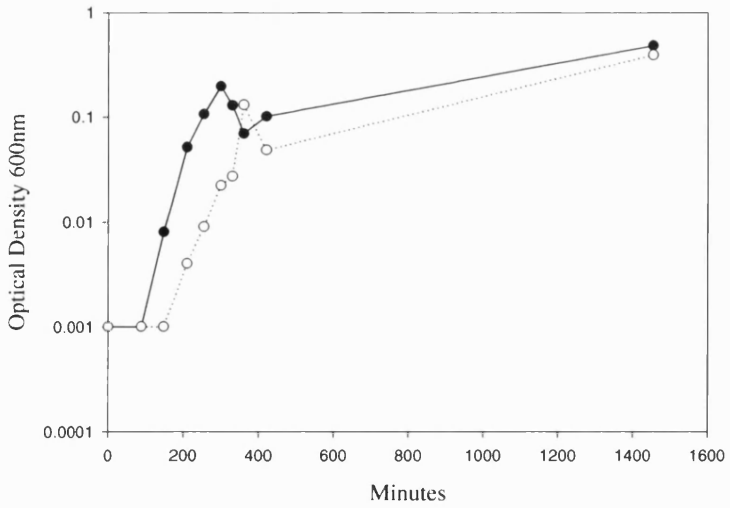


Figure 4.18 – Planktonic growth of *B. anthracis* Sterne under aeration at 37°C in magnesium-limited CDM(20) 10 μM $FeSO_4$ (●) and phosphate-limited CDM(20) 10 μM $FeSO_4$ (○), (n=1).

Since *B. anthracis* cultures were produced under each of the nutrient limitations, toxin assays were completed on each of the cultures. Both PA and LF ELISA based assays were performed to determine if the levels of these toxins or their relative ratios varied under nutrient limitation.

Unfortunately, only background or very low levels of PA and LF were detected in any of the cultures at the time points studied despite the assay being sensitive to ng concentrations of toxin. These toxin levels were vastly reduced in comparison to the levels seen in the anthrax vaccine process, implying that either the *B. cereus* CDM(20) medium is inhibitory for *B. anthracis* toxin production, or maximal toxin production occurred in between the 7 hour and 24 hour time points where no samples were taken due to logistical problems.

Despite these low levels of toxin production, the 7 hour time point taken did show a slight difference in toxin levels between the various samples. The CDM(20) with additional iron showed the highest levels in LF (4.50 ng) and the second highest levels in PA (2.30 ng) whilst CDM(20) without additional iron contained the highest amounts of PA (4.54 ng) and the second highest amounts of LF (2.24 ng). It is therefore possible that these media support the highest levels of toxin production during the peak phase and therefore oxygen, phosphate and magnesium limitation of *B. anthracis* media decrease toxin production. However, since only one set of growth curve samples were available for toxin testing (due to category 3 restrictions), it is not possible to state whether these results are statistically significant.

4.7 Discussion

4.7.1 Influence of medium on *B. cereus* toxin production

Previous studies investigating the production of enterotoxins by *B. cereus* have shown that the amount of enterotoxin expressed in cultures is dependant on the culture medium. Studies have shown that addition of 1% w/v glucose or starch to Brain Heart Infusion (BHI) leads to a significant increase in toxin production (Garcia-Arribas and Kramer, 1990). In addition, a separate study has also shown that addition of 1% glucose to BHI or 1.6% glucose to infant milk formula led to higher enterotoxin levels than cultures containing either higher or lower amounts of glucose (Rowan and Anderson, 1998). Enterotoxin levels have also been found to be increased in the presence of either fructose or lactose (Sutherland and Limond, 1993). Therefore, these results indicate an importance of a carbon source for maximal toxin production, inferring that cells grown under carbon (glucose) limitation are likely to have lower enterotoxin levels than cells grown in complete media.

Unfortunately due the presence of amino acids in the CDM(20) medium, low-density glucose limitation could not be achieved in this study. Therefore the influence of glucose limitation on enterotoxin levels was not investigated since the high-density glucose-limited cultures were also on the threshold of being oxygen-limited and hence any differences in toxin levels seen may not have been solely due to carbon limitation. The development of CDM(20) did however allow studies into the effects of phosphate, magnesium and iron limitation on toxin production. As can be seen from the results, vast differences in enterotoxin levels were found under phosphate and magnesium limiting conditions in comparison to the complete medium. These differences can be partially explained by differences in the optical densities of the cultures, especially at the later time points. However, even with cell density taken into account, the phosphate and magnesium-limited cultures still showed much lower amounts of HBL toxin.

In contrast to these results, studies with the closely related *B. thuringiensis* have found that whilst the imposition of nutrient limitation influenced the yield and morphology

of spores, nutrient limitation in the form of glucose, phosphorus, magnesium or phosphate limitation had no influence on crystal (a condensed form of the *B. thuringiensis* toxin) formation (Sakharova *et al.*, 1984). Other studies into the effects of nutrient starvation on toxin and virulence factor formation in other bacteria, including some members of the *Bacillus* family, have shown that nutrient limitation does influence toxin levels (Agata *et al.*, 1999; Hanlon *et al.*, 1982). These results therefore indicate that nutrient limitation has a different effect in various cell types, this may be explained by a cells requirement for vital chemicals required in order to produce the virulence proteins or differences in the regulation of the toxin and virulence factors.

4.7.2 Regulation of *B. cereus* toxin genes under nutrient limitation

As mentioned previously (see section 1.5.6), HBL production in *B. cereus* is regulated by PlcR. It has been reported that the transcription of *plcR* is activated at the end of the vegetative phase when cells are grown in nutrient rich media. In addition, it has also been shown that when cells are cultured in a sporulation specific medium, the transcription of *plcR* is not activated, indicting a medium dependant expression of *plcR* (Lereclus *et al.*, 2000). This regulation correlates with the results seen in this study, where maximal toxin production was found in the complete nutrient rich media and lower levels were found in the nutrient limiting, sporulating conditions. Overall these data imply that nutrient limitation influences the activation of *plcR* and the downstream virulence factors, as seen in this study.

In *B. thuringiensis* this medium dependant *plcR* regulation is controlled by Spo0A, the sporulation regulator and studies have shown that a *Spo0A* mutant over expresses PlcR when cells are cultured in a sporulation specific medium (Lereclus *et al.*, 2000; Slamti and Lereclus, 2002). Due to the close genetic relationship between *B. thuringiensis* and *B. cereus*, it is likely that a similar regulation pathway also occurs in *B. cereus* indicating that enterotoxin production is at least partially regulated by the sporulation pathway. This is in contrast with the *B. cereus* emetic toxin. Studies have shown no direct correlation between sporulation and emetic toxin production despite

maximal toxin production occurring at the end of the logarithmic phase, indicating that enterotoxin and emetic toxin are regulated via different pathways (Häggblom *et al.*, 2002).

The growth of *B. cereus* under specific nutrient limitations in this study has led to vastly different toxin yields and profiles. Since the spore properties of *Bacillus* cells have been previously found to vary under different nutrient limiting conditions (as described in sections 1.6.4 and 3.5) and the enterotoxin regulation is believed to be incorporated with the sporulation regulators, it is likely that the apparent differences seen in the toxin levels in this study are due to differences in the sporulation regulators as previously described.

4.7.3 Influence of culture conditions on *B. cereus* toxin production

In addition to understanding the effects of media composition on toxin levels, the influence of culture conditions on *B. cereus* toxin production is also an important issue. Previous studies with *B. cereus* have shown that the pH of the culture influences enterotoxin production, with higher toxin levels found in cultures where the pH is adjusted from low acid to low alkaline (Garcia-Arribas and Kramer, 1990). In addition, temperature has also been shown to have a major role in influencing toxin production. Studies have shown that a number of *B. cereus* strains are capable of growing and expressing toxins at temperatures below 15°C (Christiansson *et al.*, 1989; Griffiths, 1990; Rowan and Anderson, 1998; van Netten *et al.*, 1990) with one strain being reported to be capable of producing toxin at 4°C (Rowan and Anderson, 1998). Specifically, HBL enterotoxin levels have been found to be highest in cultures grown at 32°C, with the maximal peak occurring during the late log and early stationary phases (Fermanian *et al.*, 1996).

Furthermore, studies have also been completed investigating the influence of oxygen on enterotoxin production. Unfortunately, these studies have lead to conflicting reports, with aeration being cited to increase enterotoxin production (Christiansson *et al.*, 1989) and to have no influence (Granum *et al.*, 1993a). Therefore the influence of

oxygen limitation was also investigated in this study. The results show that the statically incubated cultures produced similar toxin levels to the aerated cultures grown in the same media with the toxin levels detected in the static cultures being much higher than the toxin levels seen under both phosphate and magnesium limitations.

Studies with the *B. cereus* emetic toxin have shown that in contrast to the results found in this study with HBL, static *B. cereus* cultures produce significantly less emetic toxin than their aerated counterparts (Finlay *et al.*, 2002) and maximal emetic toxin production occurs in shaken cultures when the dissolved oxygen tension falls below 60-70 bppm. This therefore indicates that whilst oxygen dependant respiration is required for emetic toxin production, maximal production occurs when little free oxygen is available. It can therefore be seen that changes in environmental conditions can lead to differences in production of both toxins, however the conditions required and the changes seen are not necessarily the same for both the emetic toxin and HBL enterotoxin.

At the genomic level, the enterotoxin levels are again regulated by PlcR, which in turn is influenced by Spo0A. Since the results in chapter 3 showed no clear difference in sporulation (and potentially Spo0A regulation), between the CDM static and CDM shaken cultures (see section 3.5), it is unsurprising that there is no significant difference in toxin levels with statically and aerated *B. cereus* cultures.

4.7.4 Influence of culture conditions on *B. cereus* protease production

In addition to studying the influence of culture conditions on toxin production, the effect of nutrient and oxygen limitation on protease production was also studied. The results from the assays showed detectable protease levels in all the conditions studied. With the maximal levels being detected in the complete CDM(20) cultures with added iron when grown under aeration. These results are unsurprising, as proteases are also believed to be regulated by *plcR* that is activated under nutrient rich and not nutrient limiting conditions (Agaisse *et al.*, 1999; Lereclus *et al.*, 2000; Slamti and Lereclus,

2002). Therefore nutrient and oxygen-limited cultures would be expected to produce less proteases, as seen in this study.

Analysis of the results found in this study has shown that the cultures that produced the maximal toxin levels also contained the maximal protease levels. This again is unsurprising since both virulence factors are controlled by plcR regulation and hence will be up-regulated under the same conditions. This joint regulation however, also means that it is possible that the production of HBL specific proteases are a regulatory mechanism for maintaining HBL toxin levels in *B. cereus* cultures. Since studies have shown that the addition of chemical agents including: the natural antibiotic compound, carvacrol (Ultee and Smid, 2001), bacteriocin AS-48 (Abriouel *et al.*, 2002), nisin (Beuchat *et al.*, 1997) and maltodextrin (Rowan and Anderson, 1997), to the *B. cereus* cultures can influence both HBL and NHE enterotoxin levels, the ability of protease inhibitors was also tested. These results showed that addition of inhibitors to *B. cereus* cultures led to increases in toxin levels under some nutrient limiting conditions, strongly inferring that the proteases produced by *B. cereus* are specific for the degradation of the HBL. This therefore further supports the notion that *B. cereus* proteases can act as a regulatory mechanism for HBL enterotoxin levels.

All of the *B. cereus* proteases currently reported have been members of the metalloprotease family or have close homology to other metalloproteases (Bach *et al.*, 1999; Ghorbel *et al.*, 2003; Grass *et al.*, 2004; Kim *et al.*, 2003b; Sierecka, 1998). In addition NHE has also been reported to be a metalloprotease inhibited by EDTA (Lund and Granum, 1999). Therefore, since the inclusion of EDTA into the protease inhibitor cocktail was based on it is ability to inhibit metalloproteases (Sigma), the effects of adding EDTA alone to *B. cereus* cultures was investigated. When EDTA was added at the same concentrations found in the protease inhibitor cocktail, a similar effect to the PIC was seen in terms of both protease and toxin levels, inferring that the EDTA present in the PIC is either responsible, or at least partially responsible for the protease-limiting effects seen in the *B. cereus* cultures.

4.7.5 Influence of culture conditions on *B. anthracis* toxin production

Since the original aims of this project were to investigate the culture conditions found in the anthrax vaccine process. The effect of nutrient and oxygen limitation on *B. anthracis* growth was also studied. Using the derived CDM, *B. anthracis* was able to grow under all of the conditions studied; however only to very low cell densities and with a high degree of clumping. The reasons for these low growth levels remain unclear. It is possible that the elimination of casamino acids from the medium has led to the omission of a vital nutrient required for *B. anthracis* growth, this is however unlikely since other studies into the growth requirements of *B. anthracis* vaccine strains have used individual amino acids (Naimanov *et al.*, 1986). It is also possible that the omission of charcoal has led to an inability to absorb inhibitory substances (Matsuhashi *et al.*, 1995). This however is also unlikely since other studies have generated a chemically defined 'R' medium (that omits both casamino acids and charcoal) that supports growth of *B. anthracis* Sterne to approximately 2 x 10^8 cfu / ml (Ristroph and Ivins, 1983). Therefore the low growth levels and clumping seen in the study must have been due to some other factor. Since both this study and the study by Ristroph *et al* used similar culture conditions (37°C under aeration) and similar time courses, the differences in growth must be due to media composition. The main differences between the 'R' medium and the CDM used in this study were the buffering agents (phosphate buffer compared with MOPSO) and medium pH. It is therefore likely that either MOPSO is inhibitory to *B. anthracis* growth or the pH of the CDM used in this study is too acidic to support *B. anthracis* growth.

However, despite the low growth levels, toxin assays for PA and LF were completed on all of the *B. anthracis* cultures. Unfortunately, these assays showed very low toxin levels under all the limitations studied, with the highest levels detected still being vastly lower than those currently seen in the anthrax vaccine production (Personal communication – Richard Sharp, HPA). It should, however be noted that no samples were taken between 7 and 24 hours and therefore toxin levels may have actually peaked at a higher level than those recorded.

The low toxin levels detected can not be explained by the low growth yield, since the growth in the vaccine production is also low (Personal communication – Richard Sharp, HPA); however the toxin levels may have been decreased due to the omission of charcoal and casamino acids from the CDM. It is possible that the charcoal may contribute to virulence factor production as previously described in *L. monocytogenes* (Ermolaeva *et al.*, 1999). It is also possible that there is a trace element in the casamino acids that is required for the formation of the toxins. These explanations are however unlikely, since studies using the 'R' medium has shown high toxin levels produced by Sterne, Vollum 1B and V770-NPI-R *B. anthracis* strains (Ristroph and Ivins, 1983). Therefore, as with the growth levels, the difference in toxin levels must therefore be due to some other factor.

When comparing CDM(20) to the anthrax vaccine medium, it can be seen that the two media vary in some of their components, as well as the concentrations of components present in both media. It is therefore likely that one or more of these variations is responsible for the lack of toxin production in CDM(20). The most notable differences between the two media are the buffering capacity and the inclusion of sodium bicarbonate. CDM(20) contains MOPSO to maintain the pH of the medium at 6.9. It is possible that this buffering capacity actually inhibits anthrax toxin production. It has been previously reported that maximal anthrax toxin production coincides with a drop in pH of the anthrax culture medium (Personal communication – Richard Sharp, HPA), hence if MOPSO prevents a pH drop it may actually lead to an inhibition of toxin production.

Sodium bicarbonate has also been shown to be an important factor in anthrax toxin production. As previously described (see section 1.3.3.4), anthrax toxins are regulated by AtxA. Studies have shown that although elevated (5% or higher) CO_2 or bicarbonate levels have no effect on *atxA* transcription, these conditions led to an increase in toxin gene and virulence factor expression. Hence it can be seen that *atxA* regulates CO_2 / bicarbonate induced genes (Dai and Koehler, 1997). Since the *B. anthracis* cultures grown in this study did not contain added bicarbonate, this lack of

elevated toxin gene expression may partially explain the low toxin yields seen in all the cultures compared with the current vaccine process and studies using the 'R' medium (that also contains added bicarbonate) (Ristroph and Ivins, 1983). It is therefore feasible that increased toxin levels would be seen with the addition of bicarbonate to CDM(20).

4.7.6 Conclusions

Overall it can be seen that nutrient limitation of *B. cereus* vastly effects enterotoxin production and to some degree protease levels. The addition of protease inhibitors and specifically metalloprotease inhibitors such as EDTA, are able to increase toxin levels in a number of nutrient limited cultures, especially those where the toxin levels have been vastly reduced as a result of nutrient limitation.When *B. anthracis* is cultured in the same nutrient limited medium as *B. cereus*, growth is possible, but at much lower levels. This therefore implies that whilst *B. anthracis* is able to grow under the same conditions as *B. cereus,* the two organisms have different requirements for optimal growth. The toxin assays on the *B. anthracis* nutrient limited cultures showed some differences depending on the medium, however since all of the cultures displayed only baseline toxin levels vastly lower that those seen in the anthrax vaccine production no conclusions can be draw from this study on the effects of nutrient limitation on anthrax toxin production.

Chapter 5 – Toxin production in *B. cereus* biofilms

5.1 Introduction

Biofilms growth has been proposed to be an important factor in the *B. anthracis* anthrax vaccine production (see section 1.1). During the growth processes *B. anthracis* has been shown to associate with the charcoal placed in the medium and pellicle formation occurs on the surface of the cultures. Disturbance of this pellicle during the incubation period results in culture supernatants with low vaccine potential (Personal communication – Richard Sharp, HPA), further indicating that adherent growth has an influence on toxin production of *B. anthracis.*

Due to the close genetic relationship of *B. cereus* and *B. anthracis* it is likely that biofilm formation also influences toxin production in *B. cereus* cultures. To date, numerous studies have investigated the ability of *B. cereus* to form biofilms (Lindsay *et al.*, 2002; Oosthuizen *et al.*, 2001; Oosthuizen *et al.*, 2002); however no studies have been completed investigating the production of virulence factors within *B. cereus* biofilms.

The aims of this study were therefore to develop methods allowing the growth of *B. cereus* biofilms in chemical defined nutrient limiting media. Studies were then completed investigating HBL enterotoxin production under these conditions.

5.2 *B. cereus* biofilms using a modification of the Bühler method

5.2.1 CDM(20) growth curves

Initial *B. cereus* biofilm studies used a modification of the reproducible nutrient depleted biofilm model developed by Bühler *et al* (Bühler *et al.*, 1998). This method involved this solidification of CDM with agar and the growth of *B. cereus* on a membrane on top of the medium. This growth method allowed the permeation of nutrients through the 0.2 μm pores in the membrane, without the translocation of the *B. cereus* cells to the agar; providing an efficient method to monitor the growth and density of biofilms over time.

Figure 5.1 shows *B. cereus* ATCC 14579 biofilm growth on solidified CDM(20). This figure clearly demonstrates that *B. cereus* is able to grow under these conditions with the expected lag phase (0-3 hours), logarithmic phase (3-10 hours) and stationary phase (10 hours onwards). In addition, Figure 5.2 also demonstrates a longer term growth curve under the same conditions. The results in this figure show that although the exponential phase ends as expected, the densities of the *B. cereus* biofilms continue to slowly increase during the stationary phase. This decreased growth rate is probably due to a decrease in nutrient availability, since the amount of nutrients diffusing from a solid medium into a biofilm have been shown to diminish with the depletion of substrates (Bühler *et al.*, 1998).

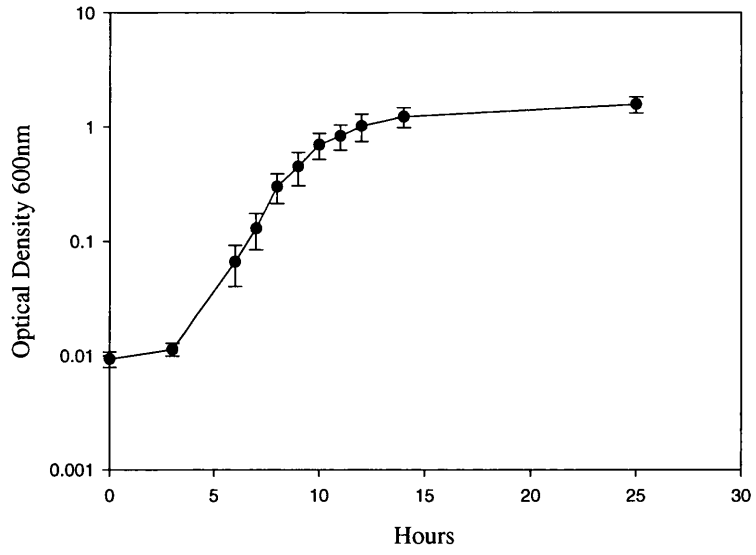


Figure 5.1 – Biofilm growth of *B. cereus* ATCC 14579 at 37°C on nitrocellulose membranes on CDM(20) agar plates, (mean ± SEM, n=3).

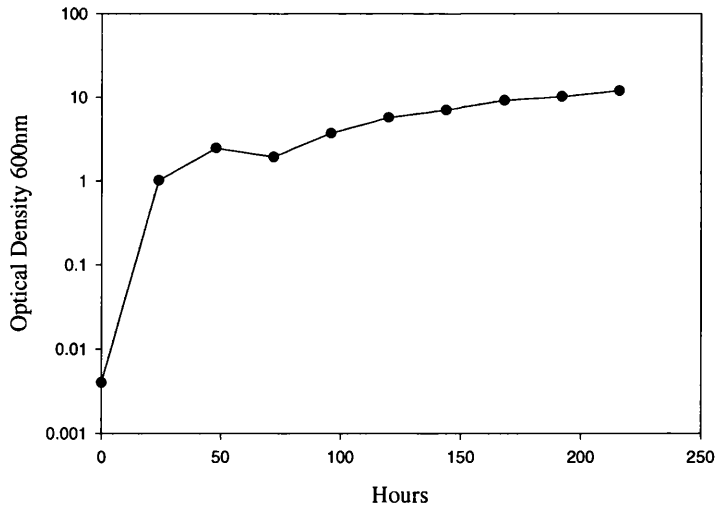


Figure 5.2 – Long term biofilm growth of *B. cereus* ATCC 14579 at 37°C on nitrocellulose membranes on CDM(20) agar plates, (n=1).

5.2.2 Nutrient limited *B. cereus* biofilms

The ability of *B. cereus* biofilms to grow using solidified CDM(20) enabled studies to be completed with nutrient limited biofilms. Therefore, nutrient limitations were completed for phosphate, glucose and magnesium. As with the planktonic cultures, these were achieved by varying the concentration of the limiting component in the medium and recording the stationary phase density of the cultures.

5.2.2.1 Phosphate limitation

B. cereus biofilms were grown on solidified CDM(20) ranging in concentrations between 0 μM and 6.4 mM added phosphate. As shown in Figure 5.3, phosphate limitation occurred at concentrations up to 500 μM phosphate after which further additions of phosphate led to no increase in biofilm density, implying that the biofilms were limited by some other factor. When no phosphate was added to the solidified CDM(20), biofilms were still able to grow at low densities, this is likely to be due to the contaminating phosphate in the glassware and medium.

5.2.2.2 Glucose limitation

Nutrient limitation was achieved for carbon by varying the concentration of glucose in the CDM(20) medium. Nutrient limitation was therefore completed using glucose concentrations between 0 mM and 55.6 mM. The results shown in Figure 5.4 show that, as with the planktonic cultures, reasonably high densities were still achieved even when no glucose was added to the medium. This growth was probably due to contaminating carbon sources in the glassware and agar, as well as the utilisation of the amino acids added to the growth medium. Despite the contamination, some degree of correlation between density and glucose concentration was seen between 0 mM and 13.9 mM added glucose, implying that high density glucose-limited biofilms are possible using these concentrations.

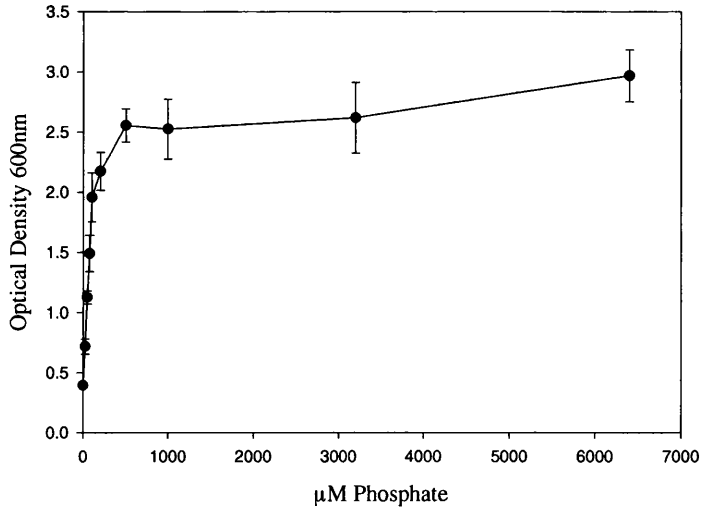


Figure 5.3 – Effect of phosphate concentration in solidified CDM(20) growth medium on stationary phase density of *B. cereus* ATCC 14579 biofilms at 37°C, (mean ± SEM, n=3).

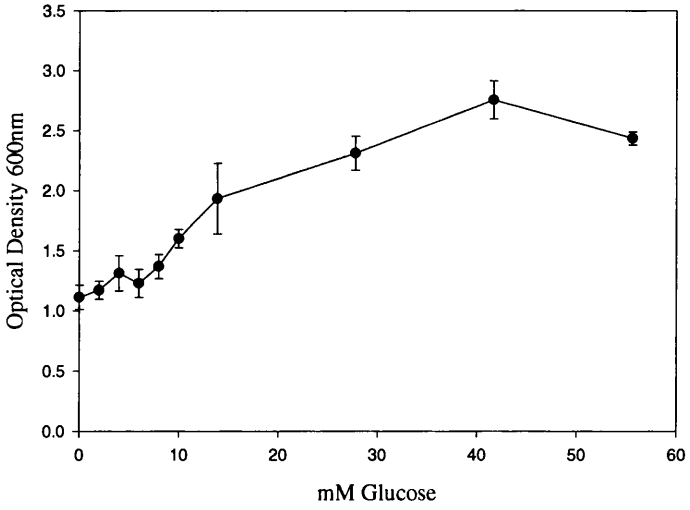


Figure 5.4 – Effect of glucose concentration in solidified CDM(20) growth medium on stationary phase density of *B. cereus* ATCC 14579 biofilms at 37°C, (mean ± SEM, n=4).

5.2.2.3 Magnesium limitation

B. cereus biofilms were also grown on solidified CDM(20) ranging in concentrations between 0 mM and 202.8 mM added magnesium. As shown in Figure 5.5, no magnesium limitation was seen at any of the concentrations used, including when no magnesium was added to the medium. This lack of magnesium limitation therefore implies that there is sufficient contaminating magnesium to support *B. cereus* biofilm growth and the biofilms produced in this experiment are inhibited by some other nutrient or factor. The cause of this contamination was therefore examined. Since magnesium limitation was achieved with the planktonic cultures (see section 3.3.3 and Figure 3.4), it was unlikely that the source of contamination was due to the CDM(20) medium or the glassware that the medium was prepared in. The use of plastic petri dishes for biofilm growth was also an unlikely source of magnesium, leaving the most obvious cause the agar used to solidify the medium. Magnesium limitation studies were therefore also completed using an ultra-pure agar source. The magnesium-limited biofilms were prepared with the same range of concentrations as above and the results are shown in Figure 5.6. As can be seen, a degree of magnesium limitation was achieved using the ultra-pure agar, with some correlation between magnesium concentration and biofilm density occurring when magnesium was added at concentrations between 0 mM and 6 mM; however, since the density of the biofilms was still reasonably high when no magnesium was added, it is apparent that the ultra-pure agar still contains contaminating magnesium, inhibiting the ability to create low-density magnesium-limited biofilms.

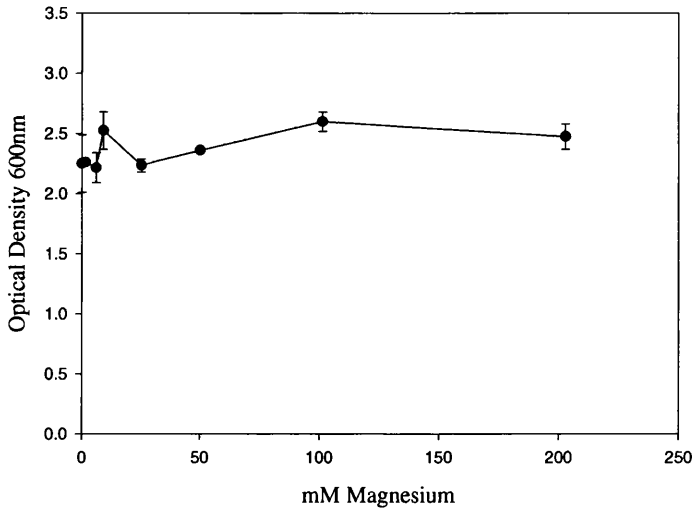


Figure 5.5 – Effect of magnesium concentration in solidified CDM(20) growth medium on stationary phase density of *B. cereus* ATCC 14579 biofilms at 37°C, (mean ± SEM, n=3).

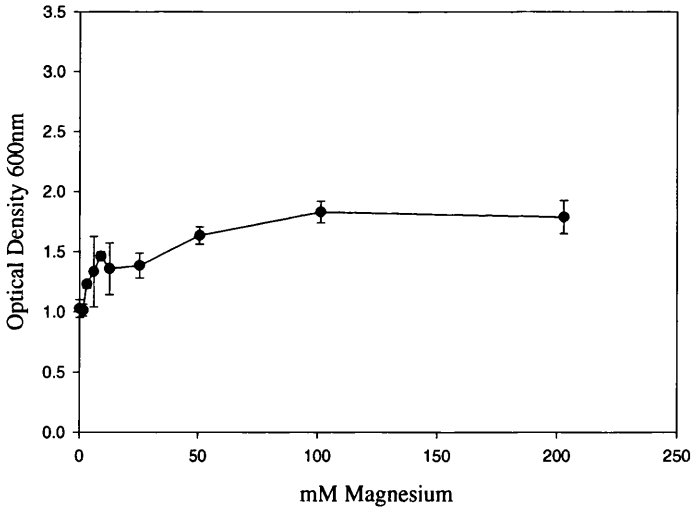


Figure 5.6 – Effect of magnesium concentration in solidified CDM(20) growth medium, using ultra-pure agar on stationary phase density of *B. cereus* ATCC 14579 biofilms at 37°C, (mean ± SEM, n=3).

5.2.3 Toxin assays on *B. cereus* biofilms (Bühler method)

Since *B. cereus* growth curves could be completed in CDM(20) and some degree of nutrient limitation could be achieved for phosphate, magnesium and glucose-limited cultures, toxin assays were completed, in order to ascertain if HBL enterotoxin was produced in *B. cereus* biofilms. In addition, since the same medium was used for both the planktonic and biofilm growth curves, comparisons of toxin levels between the two culture conditions could also be achieved.

When toxin assays were completed on the resuspended biofilm solutions from the CDM(20) growth curves and the nutrient limited biofilms, no toxin was detectable in any of the samples. Therefore, in addition, toxin assays were also completed using the washed agar samples; however these also yielded no detectable toxin levels. These data therefore suggest that either there is a lack of toxin production in the *B. cereus* biofilms, or the growth model used led to an inability to recover toxin from either the membrane or the agar lattice. Toxin control assays were therefore completed using the enterotoxin control to determine if toxin could be recovered from the biofilm growth model and detected in the toxin assays. The results from these control experiments showed that when the membranes (on solidified CDM) were inoculated with either *B. cereus* or 128 ng enterotoxin control and incubated at 37°C for 48 hours, no toxin could be detected from either the re-suspended membrane solution or the washed agar. This therefore infers that toxin may still be produced in the biofilm growth model, but is undetectable and unrecoverable given the culture conditions. Therefore, a further biofilm growth model was developed as section 5.3.

5.3 *B. cereus* biofilms using the 'floating biofilm' method

Due to the inability to recover enterotoxin from the Bühler biofilm method (described in section 5.2), a further modification to the assay was introduced. Instead of solidifying the media with agar, the membranes were added directly onto the liquid medium. Since control experiments demonstrated that the membranes were capable of floating on the media with out any mixing of the inoculum and media (due to the hydrophobic edge on the membranes), a growth curves was completed for *B. cereus* biofilms floating on CDM(20). As shown in Figure 5.7 *B. cereus* biofilms are able to grown under these conditions. The plot also once again demonstrates the typical phases of bacteria growth, with the lag phase occurring between 0 and 6 hours, the exponential logarithmic phase occurring between 6 and 12 and the stationary phase occurring after 12 hours.

Since no agar was used in this biofilm model, enterotoxin assays were completed in order to ascertain if the agar used in the previous method was responsible for the low enterotoxin recovery from the biofilms. Toxin assays were therefore completed on the biofilm growth curve samples (using both the medium and the re-suspended membrane solutions); however no toxin was detectable in any of the samples. This therefore infers that either no toxin is produced by *B. cereus* under biofilm conditions or proteases are present that are degrading the enterotoxin.

Therefore, in order to understand the influence of proteases on toxin levels, protease assays were also completed on the biofilm growth curve samples. These assays showed no protease activity in either the media or the re-suspended membranes samples inferring that the lack of toxin seen in the biofilms is not due to protease activity.

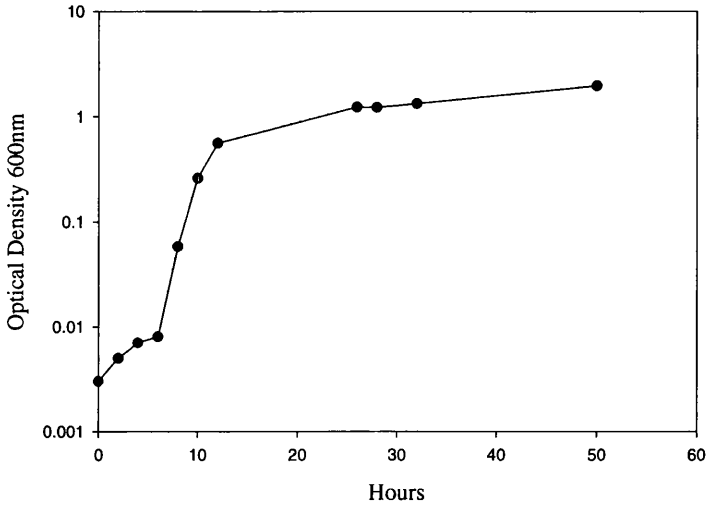


Figure 5.7 – Biofilm growth of *B. cereus* ATCC 14579 at 37°C on nitrocellulose membranes floating on CDM(20), (n=1).

It is possible that both proteases and enterotoxins are produced in the *B. cereus* biofilms, but are unrecoverable from the nitrocellulose membranes. Therefore, enterotoxin control assays were also completed. In these assays, enterotoxin control (512 ng) was inoculated both onto the membrane and directly into the medium. Samples were then incubated at 37°C for 2 hours and then prepared for toxin assay analysis. The toxin assays demonstrated that although toxin could be recovered when inoculated directly into the media, the equivalent amount of toxin could not be detected when it was added directly onto the membrane. This therefore infers that any toxin produced is binding to the nitrocellulose membrane and is not recovered for detection in the toxin assays. For this reason, this nitrocellulose membrane based biofilm method was deemed unsuitable as a model for preparing samples for enterotoxin assays. Therefore a further biofilm model was introduced for *B. cereus* growth as described in section 5.4.

5.4 *B. cereus* biofilms using the O'Toole method

5.4.1. Benefits of the O'Toole method

Numerous biofilm studies have used 96 well plates through modified versions of the O'Toole method (O'Toole *et al.*, 1999). The use of a 96 well plate has been shown to be advantageous due to the ability to run numerous replicates simultaneously and control the growth medium. Also, the addition of a small volume of media to a 96 well plate has also been shown to provide easy recovery of extracellular products produced by biofilms. Therefore, from the perspective of this study, the use of microtitre plates would allow the use of defined media for biofilm growth and ease of sample preparation for toxin assays.

5.4.2 CDM(20) growth curves

B. cereus biofilms were therefore completed as described in section 2.6.3 using CDM(20), (Figure 5.8). Statistical analysis reveals a significant difference between the medium controls and the samples ($p=0.018$, $z=-2.366$), however since the control optical densities were higher than the sample at each time point, it can be seen that this is not a clinically relevant difference. It was therefore assumed that either no biofilm growth was occurring with *B. cereus* using this method, or any biofilms formed were being destroyed by the harsh washing conditions used.

B. cereus biofilms were therefore also completed in CDM(20) using submersion as a washing method, as this was believed to be less likely to detach any biofilms formed. The results from these experiments are shown in Figure 5.8-B and as can be seen, the use of a second washing method did not led to an increase in *B. cereus* biofilm formation ($p=0.344$, $z=-0.946$), inferring that biofilms do not form under these conditions.

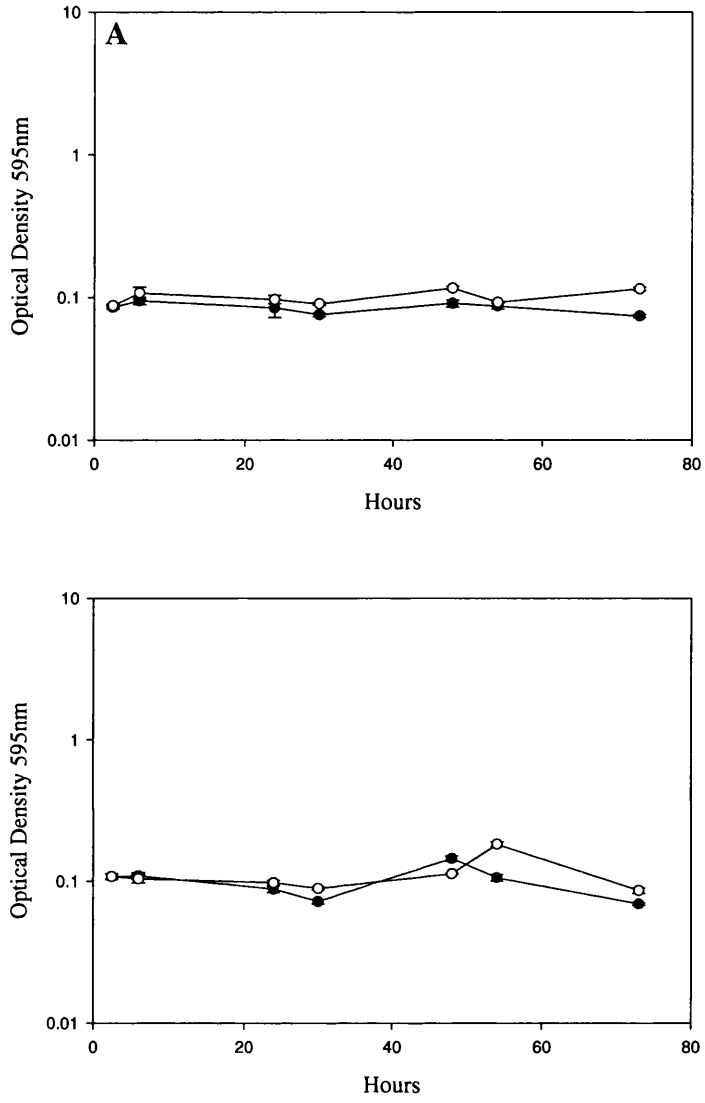


Figure 5.8 – Crystal violet staining of polystyrene 96 well plates following growth at 37°C of *B. cereus* ATCC 14579 in CDM(20) (●) or medium alone (○). Plates were washed using **A** - a 200 µl Gilson pipette and **B** – complete submersion. There was no evidence of biofilm growth, (mean ± SEM, n=6, intra-assay error).

5.4.3 Comparison with Hamon and Lazazzera

Biofilms using the O'Toole method have been previously reported for *Bacillus* species, since Hamon and Lazazzera described the use of the O'Toole method for the growth of *B. subtilis* (Hamon and Lazazzera, 2001). Their methods were therefore analysed in order to ascertain whether they included any modifications in their experiments that might lead to an increase in adherence of the *B. cereus* biofilms.

5.4.3.1 PVC plates

One of the major differences described was the use of polyvinylchloride (PVC) microtitre plates in place of the standard polystyrene plates. Therefore *B. cereus* growth curves were completed using PVC plates. The results, shown in Figure 5.9, demonstrate that once again, no *B. cereus* biofilm growth was achieved under the conditions described ($p=0.786$, $z=-0.271$).

In addition, *B. cereus* biofilms and a medium control were also crystal violet stained after a prolonged incubation (10 days) at 37°C on PVC plates. The results showed little difference between the *B. cereus* cells (OD 0.088 ± 0.009, n=6 ± SEM) and the medium control (OD 0.083 ± 0.004, n=6 ± SEM), inferring that the lack of biofilm growth seen in the growth curves is not due to an extended time taken for adherence to occur.

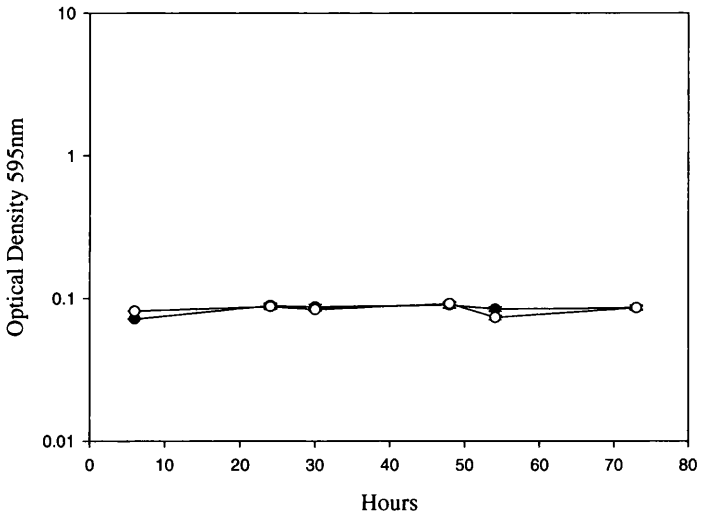


Figure 5.9 – Crystal violet staining of PVC 96 well plates following growth at 37°C of *B. cereus* ATCC 14579 in CDM(20) (●) or medium alone (○). There was no evidence of biofilm growth, (mean ± SEM, n=6, intra-assay error).

5.4.3.2 Biofilm inoculum and growth medium

In addition to modification of the plates used, the biofilms completed by Hamon and Lazazzera were also prepared using logarithmic phase cells as an inoculum. *B. cereus* biofilms were therefore also completed using logarithmic phase cells as an inoculum. The results (Figure 5.10) show that, *B. cereus* biofilm growth was still not achieved ($p=0.593$, $z=0.535$). Therefore, in addition, biofilms were also prepared using a number of other media, since the CDM(20) used in this study may have been inhibitory to biofilm formation. Growth curves were therefore completed using LB and the results are also shown in Figure 5.10. The results show higher levels of crystal violet staining with the LB grown *B. cereus* biofilms in comparison with the CDM(20) cultures, although the same difference also applies for the medium controls, hence no biofilm growth was seen with the LB cultures ($p=0.109$, $z=-1.604$).

The method described by Hamon and Lazazzera also involved the use of a supplemented LB medium for the growth of *B. subtilis*. The exact reasoning for the supplementation of a complex medium was not made clear, but as a final attempt, this medium was also prepared for the growth of *B. cereus*. The results (Figure 5.11) show a significant difference between the *B. cereus* samples and the control ($p=0.017$, $z=-2.380$), however, since biofilms were not visually apparent, it was deemed that biofilm growth had not occurred to the degree where further studies could be completed on the samples and hence this method was not used for further studies with *B.cereus.*

As a final comparison, biofilms were also prepared for *B. subtilis* using PVC plates, logarithmic phase inoculums and supplemented LB. The results for these biofilms are shown in Figure 5.12 and display a significant increase in the OD of the *B. subtilis* cells over time in comparison to the medium control ($p=0.043$, $z=-2.023$). This therefore infers that although this is a valid method for biofilm studies with some species, dense *B. cereus* biofilms are not possible using this model and therefore some other method must be used to evaluate toxin production in *B. cereus* biofilms.

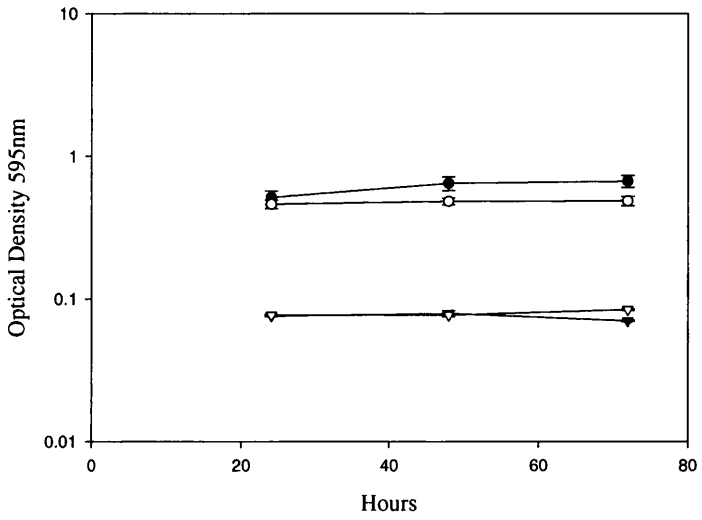


Figure 5.10 – Crystal violet staining of PVC 96 well plates following growth at 37°C of *B. cereus* ATCC 14579 after inoculation with logarithmic phase cells in either LB (●) or CDM(20) (▼) and LB(○) or CDM(20)(▽) medium alone, (mean ± SEM, n=6, intra-assay error).

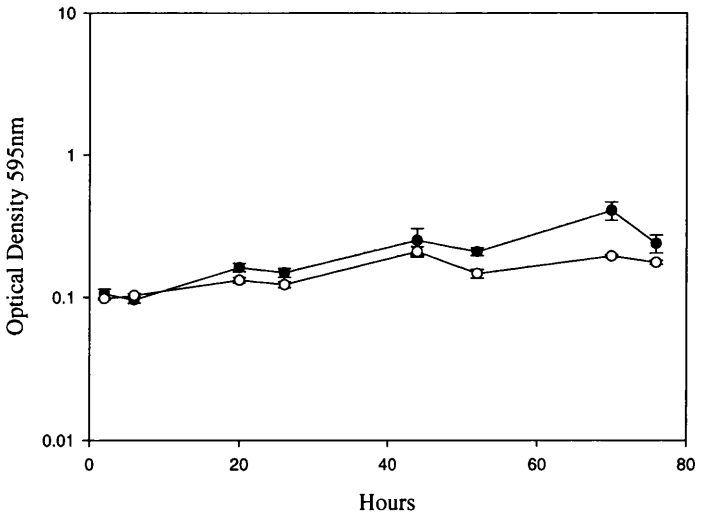


Figure 5.11 – Crystal violet staining of PVC 96 well plates following growth at 37°C of *B. cereus* ATCC 14579 after inoculation with logarithmic phase cells in supplemented LB (●) or medium alone (○), (mean ± SEM, n=6, intra-assay error).

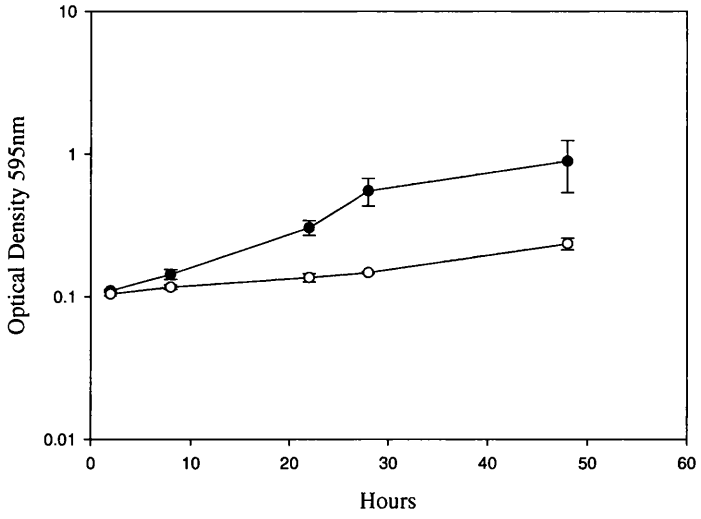


Figure 5.12 – Crystal violet staining of PVC 96 well plates following growth at 37°C of *B. subtilis* NCTC 3610 after inoculation with logarithmic phase cells in supplemented LB (•) or medium alone (○), (mean ± SEM, n=6, intra-assay error).

5.5 Quantitative real time RT-PCR using Taqman

Due to the inability to obtain biofilm samples for analysis in enterotoxin assays, production of HBL toxin in *B. cereus* biofilms was assessed by measuring mRNA levels through the use of quantitative real time RT-PCR reactions. Since the Oxoid enterotoxin test is based on the detection of L_2, primers and probes were prepared for the detection of *B. cereus HblC*, the gene encoding the L_2 protein of the enterotoxin complex.

5.5.1 Principles of the Taqman assay

The Taqman assay provides a system for the detection and analysis of RNA. The process is based on standard RT-PCR reactions, with the RNA sample being reverse transcribed to cDNA, followed by the amplification of the cDNA. In addition, the Taqman assay also includes the use of a probe containing a fluorescent dye that allows real time detection of the PCR products. The Taqman probe is designed to bind to the cDNA in between the two primers and includes a reporter dye on the 5' end and a quencher dye on the 3' end. When the probe is intact, the close proximity of the two dyes prevents fluorescence by the reporter. The Taqman probe is also designed with a melting temperature that is 10°C higher than that of the primers and hence can bind to the cDNA before the primers. Fluorescence is initiated by the 5'-3' exonuclease activity of Taq DNA polymerase. After binding of the primers to the cDNA, polymerisation of the primers occurs via Taq DNA polymerase. Upon reaching the binding site of the Taqman probe, strand displacement and cleavage occurs releasing the reporter dye from the quencher, leading to fluorescence. The intensity of fluorescence is then measured by a Sequence Detection System (SDS). This release of the reporter dye occurs with each subsequent cycle of the PCR reaction and therefore the increase in fluorescence directly correlates with the accumulation of PCR products.

5.5.2 *B. cereus* mRNA preparation

RNA extractions were initially completed on *B. cereus* cultures grown in LB overnight in order to ascertain if the RNA extraction method used was viable. Therefore, once the RNA extraction had been completed, RT-PCR and PCR reactions were run as controls. The results of these reactions (as shown by electrophoresis in Figure 5.13), show products for all of the RT-PCR reactions where the extracted template was added. By comparison, no product was present in any of the PCR reactions. This therefore infers that the RNA extraction was successful and the samples were not contaminated with DNA. In addition, it can also be seen that the RT-PCR reactions were completed using two sets of primers previously described for the detection of *B. cereus HblC* (Guinebretière *et al.*, 2002; Phelps and McKillip, 2002). It can be seen that both of these primers sets were successful, with the strong bands being visible for both reactions.

Since the RNA extraction method was therefore deemed to be viable, RNA extractions were also completed on *B. cereus* biofilm samples prepared using the floating biofilm method (as described in section 5.3). A growth curve was then completed using CDM(20) (see Figure 5.7) and RNA extractions were completed according to section 2.7.4. In addition, RNA extractions were also completed on *B. cereus* planktonic growth curve samples grown in CDM(20). These were completed as a positive control and for comparison between the Oxoid assay and Taqman assay.

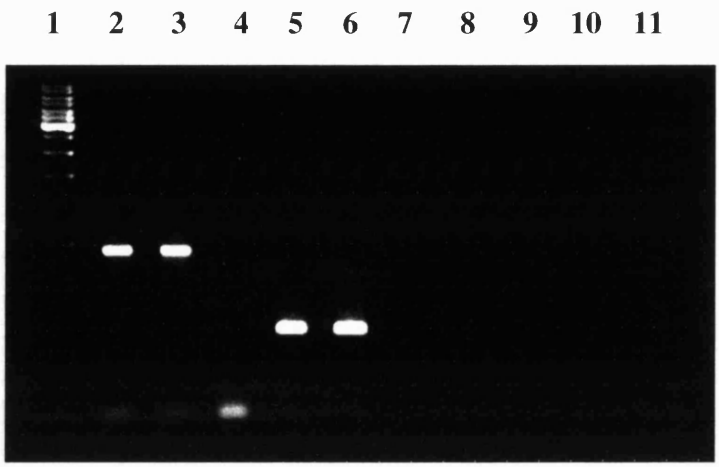


Figure 5.13 – Agarose gel analysis of RT-PCR and PCR reaction products using RNA extracted from *B. cereus* overnight cultures. Lane 1 – 1 kb ladder, lanes 2 & 3 RT-PCR reaction products using *HblC* forward and reverse primers, lane 4 as lanes 2 & 3 without template RNA, lanes 5 & 6 RT-PCR reaction products using RT forward and reverse primers, lane 7 as lanes 5 & 6 without template. Lanes 8 & 9 PCR reaction products using *HblC* forward and reverse primers and lanes 10 & 11 PCR reaction products using RT forward and reverse primers.

5.5.3 Taqman assay optimisation

In order to complete Taqman assays on the *B. cereus* growth curve samples, a series of optimisation assays had to be completed for the Taqman reactions. The first optimisation was completed for the RNA template. The RNA extracted from the overnight LB *B. cereus* cultures was diluted 10^1 – 10^4 fold in sterile water and RT-PCR reactions were completed as standard. The products were then ran on agarose gels and the final dilution where a product was still visible (10^3) was selected as the dilution factor to be used for the Taqman optimisation reactions.

The Taqman reaction mixes were then also optimised as shown in Figure 5.14. Reaction mixes were prepared with varying concentrations of both the forward and reverse Taqman primers. Taqman assays were then completed and the combination of primers giving the lowest CT value with minimal error (6 μM forward primer and 6 μM reverse primer) was then chosen for all of the remaining Taqman assays.

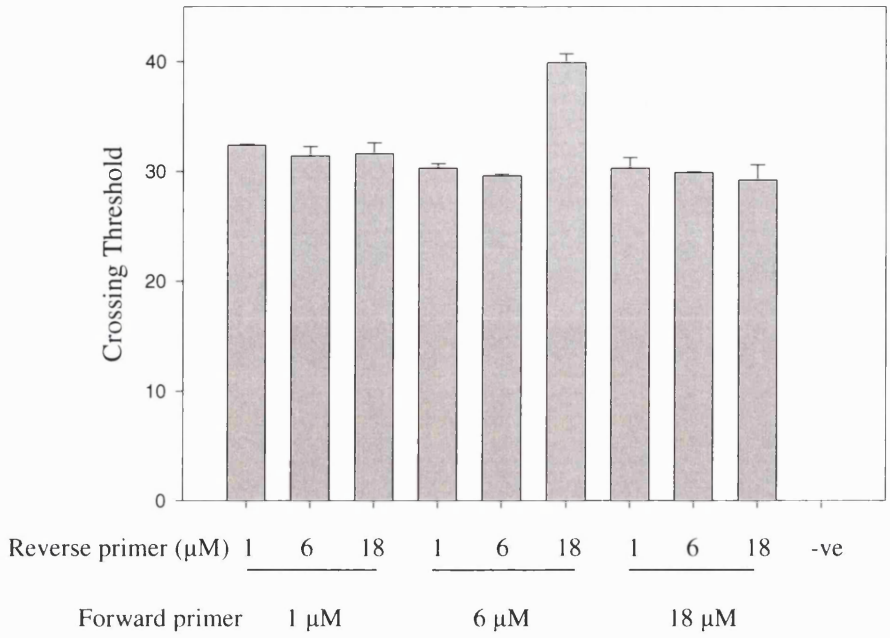


Figure 5.14 – Average crossing thresholds of Taqman reactions on *B. cereus* RNA control using varying μM concentrations of forward and reverse primers, (mean ± SD, n=3).

5.5.4 Copy number calibration

Since quantification of the Taqman assay was required, a calibration curve was established between *HblC* copy number / ml and Crossing Threshold (CT) as detected by Taqman. Cloning was therefore completed on the *B. cereus HblC* gene.

In order to achieve this, *B. cereus* was grown on LB-agar overnight and a colony was picked and incubated at 95°C for 10 minutes, in order to prepare template DNA. PCR reactions were then completed using this template and the previously designed *B. cereus HblC* primers, in a variety of combinations (Guinebretière *et al.*, 2002; Phelps and McKillip, 2002). From the results (see Figure 5.15) it can be seen that a PCR product was generated for all the combinations of primers used; however the largest product was produced when PCR reactions were completed using the RT-forward primer and *HblC*-reverse primer. This combination was therefore used for further studies.

TOPO cloning and colony screens were then completed using the PCR product selected. Minipreps were then completed on one of the positive clones and an enzyme digest was completed in order to further confirm that the PCR product had been successful inserted into the TOPO plasmid. Since the results from this enzyme digest (see Figure 5.16) showed the expected band corresponding to the *HblC* insert, the concentration of the DNA preparation was calculated and the sample was diluted for use in a Taqman Calibration curve.

The miniprep sample was serially diluted between $10^0 - 10^6$ copy numbers per ml and a Taqman assay was completed. The results from this assay are shown in Figure 5.17. As can be seen, a strong linear correlation was obtained between copy number and CT, with an r^2 value of 0.987, this was then used as a calibration curve for further assays.

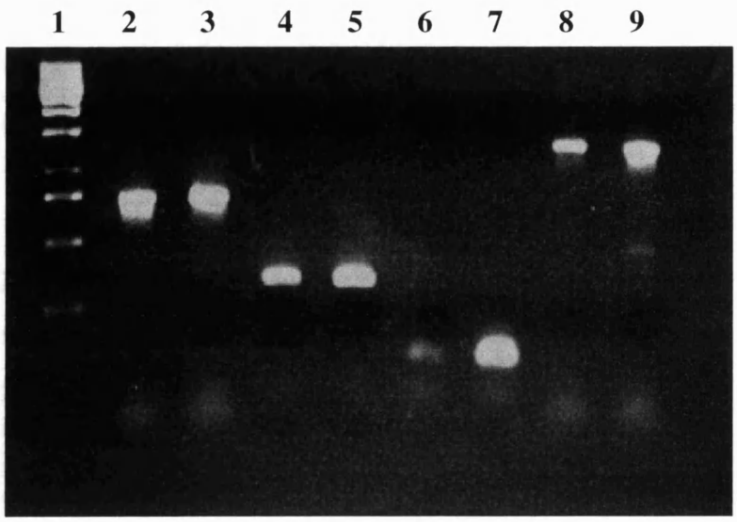


Figure 5.15 – Agarose gel analysis on PCR products using *B. cereus* template DNA and a variety of primer combinations. Lane 1 – 1 kb ladder, lanes 2 & 3 PCR reaction products using *HblC*-forward and *HblC*-reverse primers, lanes 4 & 5 PCR reaction products using RT-forward and RT-reverse primers, lanes 6 & 7 PCR reaction products using *HblC*-forward and RT-reverse primers and lanes 8 & 9 PCR reaction products using RT- forward and *HblC*-reverse primers.

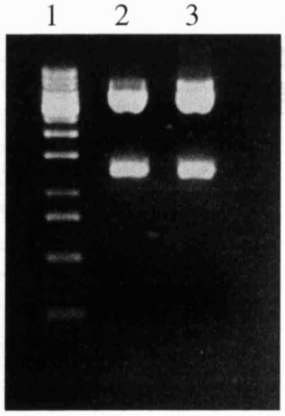


Figure 5.16 – Agarose gel analysis on enzyme digest products from miniprep samples. Lane 1 – 1 kb ladder and lanes 2 & 3 enzyme digest products.

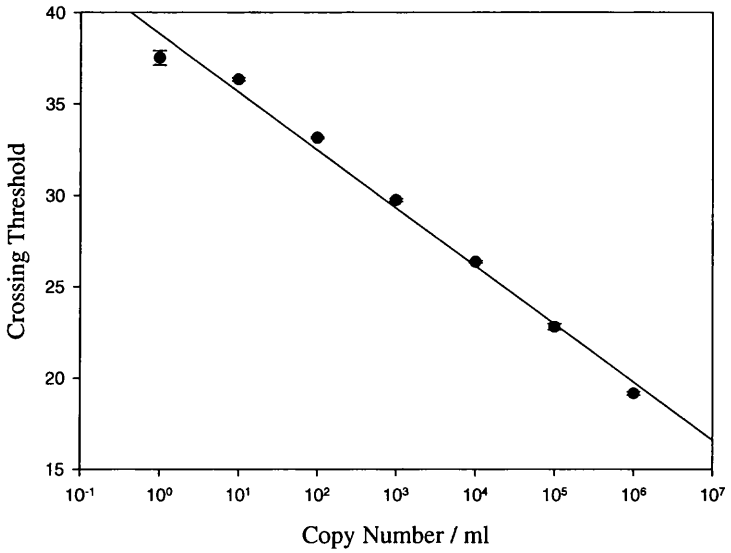


Figure 5.17 – Taqman calibration curve between *B. cereus HblC* copy number / ml and crossing threshold using 6 μM forward and 6 μM reverse primer concentrations. Linear line depicts linear regression with a 0.987 r^2 value, (mean ± SEM, n=3).

5.5.5 Taqman assays on *B. cereus* growth curve samples

Once the Taqman assay optimisation had been completed, Taqman assays were performed on the extracted RNA samples from the planktonic *B. cereus* growth curve (see Figure 5.18). As expected, *HblC* levels increase for the first 4 hours of cultures, with the levels then peaking and remaining reasonably level for the remainder of the time course, decreasing slightly at the 26 hour time point. This profile differs from the enterotoxin levels shown in Figure 4.1; however this is not unexpected. The production of mRNA is a prerequisite for translation and production of the HBL enterotoxin. Therefore, it is expected that the mRNA levels will peak before the enterotoxin protein levels, explaining the discrepancy in the 4 hour and 6 hour peaks. Also where as the HBL enterotoxin levels decrease over time due to the breakdown of the toxin by proteases etc, the mRNA is not broken down in a similar manner and hence the levels remain high throughout the rest of the time course, despite the fact that mRNA production has ceased.

Since the results for the planktonic growth curve were as expected, it was assumed that the Taqman assay was a valid indicator for HBL enterotoxin production in *B. cereus* cultures. Therefore Taqman assays were also completed on the biofilm samples. The results, as shown in Figure 5.19, clearly show the production of *HblC* mRNA over time in the *B. cereus* biofilms, strongly indicating that HBL enterotoxin is produced by *B. cereus* under biofilm growth conditions. The profile for the biofilm *HblC* levels is also similar to the planktonic culture, however with the biofilm cultures the *HblC* levels rise for the initial 8 hours before mRNA production ceases and the levels plateau out. Since the mRNA levels are expected to peak before the enterotoxin levels, it is therefore likely that the *B. cereus* biofilm enterotoxin levels peak at around 10 hours before slowly declining.

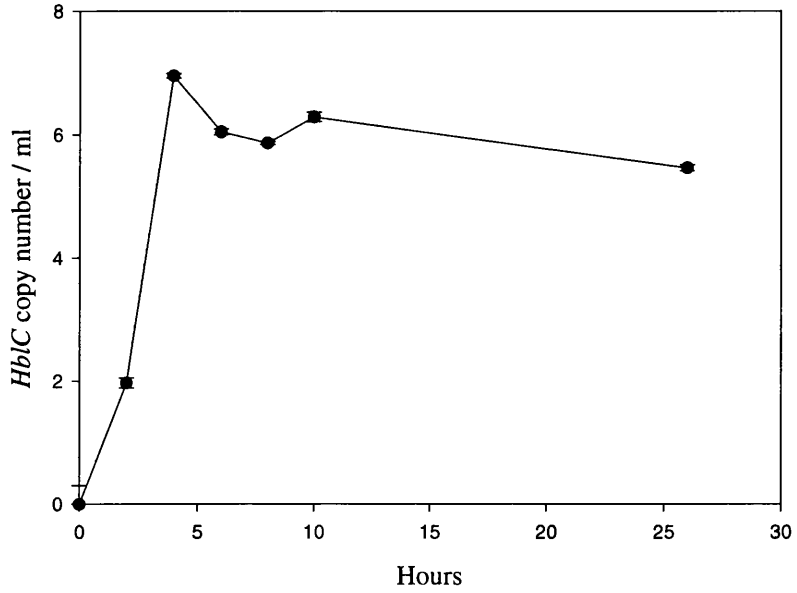


Figure 5.18 – *HblC* mRNA levels in planktonic *B. cereus* cultures grown in CDM(20) at 37°C under aeration, (mean ± SEM, n=3).

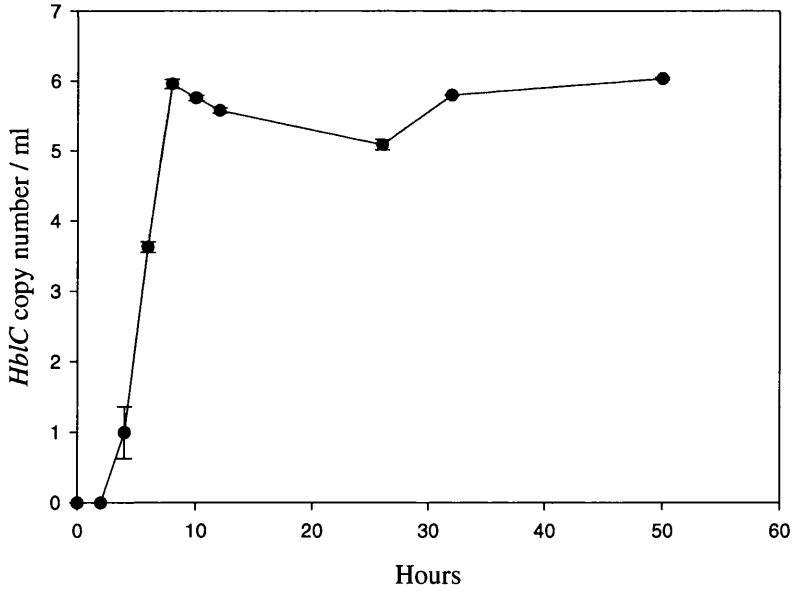


Figure 5.19 – *HblC* mRNA levels in biofilm *B. cereus* cultures grown in CDM(20) at 37°C, (mean ± SEM, n=3).

5.6 Discussion

As described previously (see section 1.1), multicellular associations have been implicated as import aspects in the anthrax vaccine production process. Therefore the influence of biofilm formation, a form of multicellular association, on toxin production was investigated in this project.

5.6.1 *B. anthracis* biofilms

Unfortunately no literature is currently available regarding *B. anthracis* biofilms and therefore little is known about *B. anthracis* biofilms or their genomic regulation. However, observations made from growth studies with *B. anthracis* in the anthrax vaccine production imply that their formation is likely due to the association of cells with charcoal particles in the medium.

It should, however, also be noted that there are other plausible explanations for the association of *B. anthracis* with the charcoal particles. Further studies may reveal that this association is not for the purpose of biofilm formation, but due to the charcoal either acting as a nutrient source or providing a chemical signal promoting bacterial growth or virulence. It is also possible that the charcoal acts as an absorber of medium components or bacterial secreted products. Studies with *L. monocytogenes* have shown that the addition of activated charcoal to a medium, whilst decreasing growth levels, leads to increased virulence factor production. It has therefore been hypothesised that the absorption of a bacterial product by the activated charcoal leads to a switching on of virulence factors genes (Ermolaeva *et al.*, 1999). This could also be the cause of the *B. anthracis* charcoal associations, partially explain the low growth yield seen in the vaccine process as well as the requirement of charcoal in the growth medium for maximal toxin production.

In addition to the influence of the charcoal particles on *B. anthracis* growth, pellicle formation has also been implicated as an important factor in the anthrax vaccine process. Pellicle formation has been previously described for other strains of bacilli (Branda *et al.*, 2004; Parisi and Antoine, 1975) and has been shown to form by *B.*

anthracis during the incubation stage of vaccine production. Studies have shown that the disturbance of this pellicle results in a vastly reduced vaccine yield, inferring that the pellicle or the surface associated growth is an important factor in terms of virulence factor production. This is probably due to density dependant signals linked to quorum sensing; however, since this pellicle forms at the liquid-gas interface, it is possible that the gas composition may also play a role in influencing toxin production (Personal communication – Richard Sharp, HPA).

5.6.2 *B. cereus* biofilms

In order to further investigate *Bacillus* biofilm formation; this study used a number of methods in an attempt to form *B. cereus* biofilms from which toxin assays could be completed. It is known that *B. cereus* is able to form biofilms since a number of previous studies have been described investigating the proteomics of biofilm formation. (Lindsay *et al.*, 2002; Oosthuizen *et al.*, 2001; Oosthuizen *et al.*, 2002; Shi *et al.*, 2004).

Unfortunately, although biofilms were formed using a modified Bühler method, toxin assays could not be completed due to a suspected adherence of HBL toxin to the nitrocellulose membranes used. Therefore further biofilm studies were completed using the O'Toole method of biofilm study.

5.6.3 Adherence and swarming

As seen from the results, *B. cereus* was unable to form biofilms on either polystyrene or PVC surfaces under the conditions used. This lack of growth has also been previously reported by Wilman, J.G *et al* (Biofilm club meeting). As mentioned previously (see section 1.7), in order for cells to form a biofilm they must be able to swarm, adhere to a surface and produce EPS in order to form robust communities. An inability in any of these traits will therefore lead to a decrease in biofilm formation and density.

When using the O'Toole method to study *B. cereus* biofilm formation, no adherence was observed in any of a variety of culture conditions studied. Previous studies investigating the adherence of Bacilli have mainly focused on the adherence of spores to surfaces. A number of reports exist referring to the role of hydrophobicity on spore adherence. Studies have revealed that increasing the hydrophobicity of both spores and surfaces promotes firmer adhesion (Faille *et al.*, 2002; Rönner *et al.*, 1990). In this study *B. cereus* biofilms did not form on either polystyrene or PVC plates, possibly due to a lack of adherence. It is therefore feasible that biofilms could have been achieved through the use of a more hydrophobic surface. In addition, it has also been shown that spores generally adhere to surfaces to a larger extent than vegetative cells (Rönner *et al.*, 1990). Studies with *B. cereus* spores have shown that they are covered in long appendages on the surface that are not present on many other *Bacillus* species (Stalheim and Granum, 2001). It has been suggested that these appendages allow the promotion of adhesion to surfaces and hence may promote biofilm formation. Therefore, since there was limited sporulation in both the CDM and LB media with the *B. cereus* strain used, it is also feasible that the high percentage of vegetative cells decreased the binding capacity of the *B. cereus* cultures used.

Studies into the swarming motility of Bacilli have focused mainly on *B. subtilis*. These studies have shown that the swarming motility of *B. subtilis* cells is affected by conditions such as nutrient status (Fujikawa, 1994). Furthermore, it has also been shown that in addition to nutrient content, cell density and the viscosity of the medium can also influence swarming (Fraser and Hughes, 1999; Harshey, 1994). It is therefore feasible that in addition to a possible inability to adhere, *B. cereus* ATCC 14579 also has an inability to swarm and sense surfaces to adhere to when grown in CDM, due to the nutrient content or viscosity of the medium. Hence, the CDM derived in this study may have lead to an inability of *B. cereus* to swarm and form biofilms on 96 well plates. However, since biofilm formation also did not occur in the complex LB medium it is unlikely that the CDM medium is the cause for the lack of biofilm formation seen in this study.

In addition to the requirement of swarming for biofilm formation, swarming has also been implicated to have a role in virulence factor production. Studies with *B. subtilis* have revealed that production of both surfactant and extracellular proteolytic activity are required for swarming and biofilm production (Connelly *et al.*, 2004)

Studies with *B. cereus* have also shown a relationship between swarming and virulence factor production. The activity of the gene, *fliY*, has been shown to be required for swarming and chemotaxis of *B. cereus* and in addition, mutations in the *fliY* gene have also been shown to lead to a deficiency in the L_2 protein of HBL, hence strains deficient in swarming were also deficient in enterotoxin production (Senesi *et al.*, 2002). From the data obtained in this study it is clear that the inability of *B. cereus* ATCC 14579 to form biofilms was not due to a defect in the *fliY* gene, since studies using the Bühler method of biofilm formation incorporated with Tacman assays, have shown an ability to detect the L_2 component of HBL in *B. cereus* biofilms. It is however feasible that *B. cereus* ATCC 14579 is unable to form biofilms using the O'Toole method due to mutations in other genes required for the swarming phenotype.

In addition to a lack of adherence or swarming, it is also feasible that *B. cereus* ATCC 14579 was unable to form biofilms due to a lack of sufficient levels of EPS production. However, in contrast to these results a recent report (Shi *et al.*, 2004) has shown biofilm growth for the ATCC 14579 strain on polystyrene plates. This study was completed using the O'Toole method with the cells grown in LB at 30°C instead of 37°C. It is therefore possible that this bacteria strain is capable of adhering and producing high levels of EPS, but only in a temperature dependant manner. This relationship between temperature and polysaccharide production has been reported for other bacteria, however only in shaken cultures and not static conditions as used in this study (Moonmangmee *et al.*, 2002).

It is also possible the sub culturing and storage conditions of the cells used with the *B. cereus* biofilm studies have varied leading to differences in the phenotype and

genotype. This has been shown for *B. subtilis* where domestic strains such as *B. subtilis* 168, form thin and relatively undifferentiated biofilms; where as wild isolates have been shown to form organised biofilms with distinct architectural features, including fruiting body like projections that extend from the surfaces of the biofilm (Branda *et al.*, 2001). If this were the case, the lack of biofilm formation on 96 well plates found in this in this study would be similar to the lack of biofilm formation found with laboratory *B. subtilis* strains and using a wild isolate may have lead to an increase in biofilm formation.

5.6.4 Nutrient limited biofilms

One of the main aims of this study was to investigate the effects of nutrient limitation within biofilms on toxin production. Studies with *B. cereus* have revealed that cells may switch from an aerobic form of growth to a fermentative mode of growth under biofilm conditions. This was shown by the up-regulation of catabolic ornithine carbamoyltransferase in biofilm cells in comparison with planktonic cells (Oosthuizen *et al.*, 2002). This fermentative mode also involves a requirement for glucose, pyruvate or the degradation of L-arginine, in place of the respiration of oxygen (Maghnouj *et al.*, 1998). Hence the nutrient requirements of *B. cereus* biofilms and the influence of nutrient limitation on biofilms, may vary from the equivalent planktonic cultures.

To fully understand the nutrient requirements of biofilms, nutrient limiting studies are required, however it should be noted that studies into single-species biofilms have shown them to be heterogeneous in terms of gene expression, due to the diffusion limitations imparted by the biofilm. This heterogeneous phenotype also leads to local variations in pH, nutrient and oxygen availability, as well as varying concentrations of bacterial metabolites (Jefferson, 2004). This means that when investigating nutrient limitation in biofilms, the exact availability of nutrients on a cell-cell basis cannot be known and is likely to vary from cell to cell.

Despite this fact, nutrient limitation studies have been achieved for biofilms as a whole. Bühler *et al* reported the generation of chemically defined glucose-limited *E. coli* biofilms and iron-limited *Burkholderia cepacia* biofilms. Further studies with these biofilms revealed, that as with planktonic cells, nutrient limitation influences protein and hence potentially toxin, production. Studies revealed that iron-limited *B. cepacia* biofilms produced a 66 kDa protein where as the iron-plentiful culture only generated very small amounts of protein (Bühler *et al.*, 1998). Furthermore, studies with Citrobacter biofilms have also revealed changes in activity and production of a phosphatase enzyme under varying nutrient limitations (Allan *et al.*, 2002).

In this study, nutrient limited biofilms were achieved for phosphate-limited *B. cereus* cultures, high-density magnesium-limited cultures and high-density glucose-limited cultures using the Bühler method. Based on the observation above, it is likely that the HBL enterotoxin in these biofilms was also influenced by the nutrient limitation, however due to the problems of detecting the toxin formed in these biofilms and a lack of time, these studies could not be completed and hence further investigations using the modified Bühler method and Tacman detection of *hblC* would be required.

5.6.5 Conclusions

It can be seen that nutrient limitation has been achieved for *B. cereus* biofilms using the Bühler method; however, it is still unclear as to whether these nutrient limitations of *B. cereus* biofilms influenced toxin and virulence factor production. Studies using the O'Toole method revealed a lack of cell adherence and hence *B. cereus* biofilm formation on either PVC or polystyrene 96 well plates, this may have been due to a lack of EPS formation due to culture conditions, a lack of swarming due to the nutrient content of the media, or a lack of adherence due to hydrophobicity or some similar factor. Depsite these problems, toxin levels were investigated in CDM complete biofilms. Quantitative real time RT-PCR reactions revealed that *B. cereus* biofilms contained detectable levels of mRNA for the L_2 component of the HBL toxin, inferring that toxin production occurs during *B. cereus* biofilm growth.

The initial aims of this project were to use *B. cereus* in order to develop procedures for biofilm studies with *B. anthracis*. Unfortunately, due to a lack of functioning facilities during the time course of this project, the influence of biofilm formation on *B. anthracis* toxin production could not be directly investigated. However, since this study has shown that biofilm growth of *Bacillus* species allows toxin production and it has been previously observed that the disruption of the pellicle in the anthrax vaccine process leads to a decrease in vaccine yield, it is likely that biofilm formation plays an important role in anthrax toxin production. In addition, it is also likely that the composition of the growth medium also influences biofilm growth and virulence factor production within the biofilm.

Chapter 6 - Concluding remarks

The original aims of this project were to understand the effects of specific nutrient limitations on the physiology and *in vitro* toxin production of *B. anthracis,* in order to inform further the current UK anthrax vaccine process. Initial work was therefore performed on the closely related pathogen, *B. cereus*, in order to develop a number of methods, assays and safe working practices for later transfer to *B. anthracis.* Unfortunately, due to a lack of functional facilities the majority of the *B. anthracis* assays could not be completed during the time course of this project and therefore the effects of nutrient limitation on *B. anthracis* physiology have not been fully investigated. Despite these drawbacks, the work completed in this project has given an insight into the effects of nutrient limitation on the closely related human food pathogen, *B. cereus.*

Studies into medium requirements of *B. anthracis* for *in vitro* PA production were initiated in the 1950s (Belton and Strange, 1954); however since then little research has been completed to further characterise the effect of the growth medium on the anthrax vaccine process. It has been proposed that the U.K. anthrax vaccine medium imposes a range of nutrient limitations on *B. anthracis* cells and studies on a range of bacteria have shown that specific nutrient limitations lead to variations in virulence factor production and gross physiology (Chaussee *et al.*, 1997; Lemes-Marques and Yano, 2004; McKenney and Allison, 1995; Ombaka *et al.*, 1983). This project was therefore completed in order to examine the influences of nutrient limitation on both *B. cereus* and *B. anthracis* toxin production in order to understand toxin production in the current UK anthrax vaccine.

In order to investigate the role of specific nutrient limitations on *B. cereus* physiology, a chemically defined medium was required for the growth of *B. cereus* ATCC 14579. This has been successfully developed in this study and defined variations on this medium have allowed studies into the effects of specific nutrient limitations to be

completed. These studies have shown that nutrient limitation influences sporulation, heat resistance and virulence factor production of *B. cereus* cells.

Studies into the sporulation of *B. cereus* ATCC 14579 revealed that complete sporulation does not occur either in CDM or complex media when cells are incubated either statically or under aeration at 37°C. Since previous studies have shown that *B. cereus* ATCC 14579 does sporulate in complex media when incubated at 30°C (de Vries *et al.*, 2004) and *B. subtilis* can sporulate in complex media at 37°C (Piggot and Hilbert, 2004), it can be seen that environmental conditions, such as temperature, influence *B. cereus* sporulation. In addition, it can also be concluded that the stress response of Bacilli is not universal across all species This divergent stress response has been previously reported for *B. cereus* and *B. subtilis,* since genomic studies have revealed the up-regulation of different genes in responses to stresses (de Vries *et al.*, 2004). Hence, it can be seen that whilst members of the *B. cereus* group are closely related they demonstrate differences in their regulatory mechanisms.

Although there was a lack of released spores in the *B. cereus* cultures, analysis of samples revealed differences in the gross morphology of the cells. It was shown that different limitations had varying amounts of influence on *B. cereus* cell morphology and where changes in cell morphology were apparent, different nutrient limitations led to differences in cell shape and phase brightness. It can therefore be concluded that specific nutrient limitations can influence the stress response of *B. cereus* and the gross physiology of the cells.

The developed CDM also allowed the influences of nutrient limitation on heat kill to be investigated. Previous studies investigating *B. cereus* heat resistance have shown that cells become more resistant to stresses after an initial exposure to a sub-lethal condition mediated by the up-regulation of σ^B (Browne and Dowds, 2001; Browne and Dowds, 2002; Periago *et al.*, 2002; van Schaik *et al.*, 2004a). The results from this study demonstrated that generally, *B. cereus* cells grown in nutrient limited media were more susceptible to heat kill at 50°C than cells grown in complete or complex media. Since it has been reported that ATP depletion and hence nutrient limitation

does not lead to an up regulation of σ^B (van Schaik *et al.*, 2004a), it can be seen that the nutrient limitations imposed in this study have not led to an up regulation σ^B, preventing the cells from adapting to a subsequent heat shock. An exception to these results was seen with the phosphate-limited cultures. These cultures demonstrated the greatest overall heat resistance despite being initially susceptible to 50°C incubations. This therefore infers that phosphate limitation leads to a bi-phasic population of *B. cereus* cells that are able to demonstrate a degree of stress adaptation. Since no specific data are available regarding the up-regulation of σ^B due to phosphate limitation, it is possible that unlike the other nutrient limitations phosphate limitation leads to the activation of σ^B.

The main focus of this study was the influence of nutrient limitation on virulence factor production. Previous studies have shown that nutrient limitation influences virulence factor production (Agata *et al.*, 1999; Ristroph and Ivins, 1983) and consistent with those reports, the results shown in this study demonstrated that whilst some limitations had very little influence of enterotoxin levels, HBL toxin levels were vastly decreased with both phosphate and magnesium limitations. Since the anthrax vaccine medium contains no added phosphate and has been proposed to be phosphate-limited, this may also be relevant to the vaccine production process. In addition, data from this study have also shown that protease levels in *B. cereus* cultures also vary with nutrient limitations; with the cells grown in compete medium producing the highest levels in comparison with the nutrient limited cultures. It can therefore be concluded that nutrient limitation of *B. cereus* does influence virulence factor production. Further studies investigating the effects of varying amounts of metalloprotease inhibitors on *B. cereus* protease and toxin levels have also revealed that protease inhibitors are able to influence toxin levels under some nutrient limiting conditions. Protease inhibitors had little influence on toxin levels in CDM complete cultures; however their addition vastly increased HBL levels in both phosphate and magnesium-limited cultures. These results therefore show that proteases produced by *B. cereus* are specific for HBL enterotoxin and may therefore be part of an enterotoxin regulation mechanism.

In addition to the studies completed on *B. cereus*, the CDM was also used to study growth and toxin production of *B. anthracis*. Unfortunately, although *B. anthracis* was able to grow in the CDM derived for *B. cereus*, only low-density cultures could be achieved and very low toxin levels were present in both the nutrient limited and complete cultures. This indicates that whilst *B. anthracis* is able to grow under the same conditions as *B. cereus*, the two organisms have different nutrient requirements. It can therefore be concluded that the results obtained from nutrient limited studies with *B. cereus* are not necessarily applicable for *B. anthracis*. This therefore suggests that *B. cereus* may not be a valid surrogate for *B. anthracis* in all situations.

The low levels of toxin production seen in the *B. anthracis* cultures could not be attributed solely to the low growth levels seen in the cultures, since growth has been reported to reach only low densities in the anthrax vaccine medium. The lack of toxin production could also not be attributed to the growth temperature, lack of casamino acids or omission of charcoal from the medium, since studies using a similar 'R' medium have previously reported high toxin levels using the same *B. anthracis* strain (Ristroph and Ivins, 1983). This therefore suggests that the low toxin levels found in the CDM cultures was due to the some factor in the growth medium. This could have been the pH of the medium or the buffer used in the medium, although it is more likely that the low toxin levels seen in this study are due to a lack of sodium bicarbonate in the growth medium. Bicarbonate has been previously shown to up regulate the toxin genes (Dai and Koehler, 1997) and it is therefore possible that addition of sodium bicarbonate to complete CDM would lead to an increase in *B. anthracis* toxin production.

Although the majority of studies completed in this project used planktonic cultures, studies were also completed using *B. cereus* biofilms due to the possibility of biofilm growth influencing the anthrax vaccine process. A number of different methods and modifications were used in this study in order to generate *B. cereus* biofilms grown on or in chemically defined media. A number of problems were encountered with the *B. cereus* biofilms, since although they were easily prepared using modifications of the

Bühler method, no enterotoxin could be detected due to the binding of HBL protein to the nitrocellulose membranes. Studies using modifications of the O'Toole biofilm method were unsuccessful due to the inability of *B. cereus* ATCC 14579 to form biofilms on plastic surfaces. This was attributed either to a lack of adherence, swarming or EPS production, although it should be noted that the same strain has been reported to form biofilms using similar techniques in other studies (Shi *et al.*, 2004). The lack of biofilm formation may have also been due to the culture conditions or mutations in the strain over time, as has been reported for *B. subtilis* (Branda *et al.*, 2001). Due to these problems, the only method available to monitor HBL levels in *B. cereus* biofilms was by the use of molecular techniques monitoring toxin mRNA levels. These studies were completed using Taqman assays that allowed quantitative real-time RT-PCR reactions to be completed on the toxin mRNA present in the biofilm growth curve samples. The results from the Taqman assays completed on *B. cereus* biofilms grown in complete CDM using a modified Bühler method, demonstrated that HBL toxin is produced in biofilms as well as planktonic cultures. However to date, it has not been determined if nutrient limitation of *B. cereus* biofilms also influences toxin levels as has been shown for both *B. cepacia* and *Citrobacter* (Allan *et al.*, 2002; Bühler *et al.*, 1998).

6.1 – Suggestion for future work

- Using antibodies against σ^B, complete a range of western blots in order to confirm whether σ^B is up-regulated under specific nutrient limitations. Also complete a range of western blots using antibodies raised against other alternative sigma factors in order to investigate if they have a role in the nutrient limitation stress response of *B. cereus.*

- Investigate the influence of charcoal on *B. cereus* and *B. anthracis* growth and toxin production.

- Refine the developed chemically defined medium for *B. anthracis* growth in order to increase growth yield and toxin production. Complete further studies investigating the effects of nutrient limitations on PA and LF production as well as study the influence of protease production on toxin levels in *B. anthracis* cultures.

- Continue *B. cereus* biofilm studies using the floating biofilm method and complete a range of nutrient limited growth curves. Using samples from these growth curves and Taqman assays, investigate if specific nutrient limitations influence *B. cereus* virulence factor production in biofilms. In addition, further refine the Taqman assays by standardising the RNA extraction levels, by either developing an additional Taqman assay measuring mRNA levels of a *B. cereus* housekeeping gene or by spiking samples with virus RNA and determining levels of recovery using Taqman assays.

- Transfer the methods developed for *B. cereus* biofilm studies to *B. anthracis,* in order to determine if biofilm growth influences toxin production in the anthrax vaccine process., as has previously been implied.

Chapter 7 - References

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Chapter 8 – Appendix

8.1 Appendix 1- UK Anthrax vaccine medium

Basal Medium

Component	Amount / Volume
Casamino acids	5.956 g
L-Cystine	0.02 g
Calcium chloride ($CaCl_2.6H_2O$)	0.0245 g
Magnesium Sulphate ($MgSO_4.7H_2O$)	0.025 g
Potassium hydroxide (KOH)	0.518 g
D-L-Serine	0.052 g
Carbon D (activated charcoal)	0.278 g
Thiamine solution (*)	0.1 ml
Concentrated Hydrochloric acid (HCl)	as required
Purified water	to 1000 ml

(*) Thiamine solution
Thiamine hydrochloride (0.167 g) + purified water to 100 ml

Basal Medium pH is adjusted to pH 6.9 using concentrated HCl
Basal medium is sterilised by autoclaving after preparation

Additional Medium

Component	Amount / Volume
Sodium hydrogen carbonate ($NaHCO_3$)	60 g
Dextrose (D+ glucose, anhydrous)	20 g
Manganese sulphate ($MnSO_4.4H_2O$)	0.01 g
Purified water	to 1000 ml

Additional medium is sterilised by filter sterilisation after preparation

Final Medium Preparation

Component	Volume
Basal Medium	450 ml
Additional Medium	50 ml